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Biosíntesis y acumulación de carotenoides en frutos cítricos y su implicación en la calidad postcosecha

Tesis Doctoral presentada por **Joanna Lado Lindner**

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- **Joanna Lado**, María Jesús Rodrigo, Paul Conje, Lorenzo Zacarías (2015). **Involvement of lycopene in chilling injury resistance in grapefruit**. *Postharvest Biology and Technology* 100, 176-186.

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Dra. Guillermina Font

Para vos Madre, un pilar siempre presente

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LISTA DE ABREVIATURAS

1-MCP-1-Metil ciclopropeno
AAPH-Dihidrocloruro 2',2'-azobis-2-metilpropionamida
ABA-Ácido abscísico
ABTS-Ácido 2,2’-azinobis (3etilbenzotiazolín)-6-sulfónico
ACC-Ácido carboxílico-1-aminociclopropano
ACO-ACC oxidasa
ACS-ACC sintasa
ADH-Alcohol deshidrogenasa
APX-Ascorbato peroxidasa
AsA- Ácido ascórbico
βCHX- β-Caroteno hidroxilasa
βLCY- β-Licopeno ciclasa
C-Cara Cara
C-Tapado/covered
CAT-Catalasa
Chl-Clorofila
CI-Daño por frío o ‘chilling injury’
CPTA- Hidrocloruro de 2-(4-tioclorofenil) trietilamina
DF-Daño por frío
DMAPP-Dimetilalil pirofosfato
DPPH-2,2-difenil-1-picrilhidrazilo
DXS- Dioxixilulosa-5-fosfato sintasa
εCHX-ε-Caroteno hidroxilasa
εLCY-ε-Licopeno ciclasa
ETR-Receptores de etileno o ‘ethylene receptors’
ERF-Factores de respuesta a etileno o ‘ethylene response factors’
FIB-Fibrilina
FW-Peso fresco o ‘fresh weight’
GGPP-Geranil geranil pirofosfato
GGPPS-Geranil geranil pirofosfato sintasa
GR-Glutatión reductasa
GSH-Glutatión
GSSG-Disulfuro de glutatión
H_2O_2-Peróxido de hidrógeno
HDR-Hidroximetil butenil difosfato reductasa
HMBPP-Hidroximetilbutenil difosfato
HPLC-PDA- Cromatografía líquida de alta resolución acoplada a detector de fotodiodos
HSP-Proteínas de choque térmico o ‘heat shock proteins’
MDA-Malondialdehído
MEP-Metil-eritritol-fosfato
MEV-Ácido mevalónico

MSH-Marsh
N-Navel
NC-No tapado o ‘non covered’
NCEDs- 9-*Z*-Epoxicarotenoide dioxigenasas
NSY-Neoxantina sintasa
ORAC-Capacidad de absorción de radicales de oxígeno o ‘oxygen radical absorbance capacity’
P-Pinalate
PAL-Fenilalanina amonio liasa
PDS-Fitoeno desaturasa
PF-Peso fresco
PIPES-Piperazina-N,N`-bis(ácido 2-etanosulfónico)
PSY-Fitoeno sintasa
ROS-Especies reactivas del oxígeno o ‘reactive oxygen species’
SAM-S-adenosil-metionina
SOAC- Capacidad de secuestro del oxígeno singlete o ‘single oxygen absorption capacity’
SOD-Superóxido dismutasa
SR-Star Ruby
TEM-Microscopía electronica de transmisión
ZDS-ζ-caroteno desaturasa

RESUMEN

La conservación refrigerada es una de las tecnologías más utilizadas para preservar la calidad postcosecha de frutas y hortalizas. Los cítricos son el principal frutal que se produce en el mundo y el transporte refrigerado es esencial para conservar la calidad y cumplir con los requisitos de los mercados. Sin embargo, los cítricos presentan una alta sensibilidad a las bajas temperaturas. Los frutos de pomelos se encuentran entre los cítricos más sensibles al frío y desarrollan manchados en la piel conocidos como daño por frío (DF), a temperaturas por debajo de 8-10 ºC. Estas alteraciones no afectan a la calidad interna del fruto, pero deprecian de forma importante su valor comercial. Por ello, es necesario conocer los factores implicados en los DF a fin de identificar posibles estrategias para un control eficiente de esta fisiopatía. Por otro lado, los carotenoides son los pigmentos responsables de la coloración de los frutos cítricos y, por tanto, su contenido y composición determina de forma importante la calidad. Con estos antecedentes, en esta Tesis Doctoral se ha abordado el estudio del papel de los carotenoides (Capítulo I), la influencia del etileno (Capítulo II) y del sistema antioxidante (Capítulo III) en la respuesta de los frutos de pomelo y su sensibilidad al DF durante la conservación refrigerada. Este objetivo se ha complementado con la caracterización de los cambios fisiológicos, moleculares y celulares que ocurren en frutos cítricos en condiciones ambientales de oscuridad (Capítulo IV) y en variedades con diferente pigmentación durante la maduración (Capítulo V).

Durante almacenamiento en frío de los frutos de pomelo blanco Marsh (MSH) y pomelo rojo Star Ruby (SR), se observó que la incidencia de DF en la variedad roja SR se limitó exclusivamente a las zonas amarillas de la piel, mientras que las zonas rojas permanecieron completamente intactas. El contenido y composición de carotenoides entre ambas zonas fue muy diferente, con el doble de carotenoides totales y especialmente 14 veces más licopeno en las zonas rojas. Para comprobar la implicación del licopeno en la tolerancia al DF de las zonas rojas, se incrementó el contenido del mismo mediante dos estrategias: inducción química y manipulación ambiental. La aplicación postcosecha de CPTA [2-(4-chlorofeniltio)trietilamina hidrocloruro], un inhibidor de la ciclación del licopeno, a frutos de pomelos MSH incrementó el contenido en carotenoides (x 7) y principalmente de licopeno (x 32) en la piel de los frutos y redujo la susceptibilidad a los DF durante la conservación posterior a 2 ºC. En una segunda aproximación se taparon los frutos de pomelo SR durante la maduración en el árbol, lo cual potenció la coloración roja de la piel, respecto a los expuestos a fotoperiodo normal, y se incrementó la acumulación de carotenoides, principalmente los carotenos fitoeno (x 3) y licopeno (x 75). Los frutos tapados, con un alto contenido en licopeno, fueron más tolerantes a los DF que los no tapados.

Se ha investigado la implicación del etileno, tanto de la biosíntesis como de la percepción, en la sensibilidad al DF en los frutos de pomelo. Los frutos de ambas variedades de pomelo manifestaron DF a partir de los 15-20 días de almacenamiento a 2 ºC y la expresión de los genes de biosíntesis de etileno (*ACS1, ACS2* y *ACO*) se estimuló exclusivamente en los frutos sensibles, por lo que estos genes podrían ser buenos marcadores de sensibilidad al frío. La aplicación de 1-MCP, un inhibidor de la acción del etileno, provocó un aumento notable de la producción de la hormona, a través de la estimulación de los genes biosintéticos, corroborando la regulación auto-inhibitoria de la síntesis de etileno en los frutos de pomelo. La expresión de genes correspondientes a receptores de etileno (*ETR*s) y factores de respuesta (*ERF*s) se estimularon por las bajas

temperaturas de conservación. Dado que la principal función de los carotenoides como antioxidantes radica en la inactivación de especies reactivas del oxígeno, principalmente el oxígeno singlete, se evaluó la implicación del sistema antioxidante en la piel de frutos sensibles y tolerantes al DF. Los frutos tapados, con un mayor contenido de licopeno y tolerantes al DF, presentaron una capacidad para inactivar el oxígeno singlete (SOAC) 2-3 veces superior a la de los frutos sensibles durante todo el período de almacenamiento. La evaluación de los sistemas antioxidantes enzimático (glutatión reductasa, superóxido dismutasa, catalasa, ascorbato peroxidasa) y no enzimático (ácido ascórbico y glutatión), reveló un papel marginal de ambos sistemas en la mayor tolerancia al DF de los frutos tapados. En su conjunto estos resultados sugieren que el licopeno podría ser el responsable de la tolerancia de los frutos de pomelo a los DF, a través de la inactivación del oxígeno singlete, indicando, a su vez, un posible papel de esta especie reactiva en el daño oxidativo por las bajas temperaturas.

En la segunda parte de esta tesis se han estudiado las bases fisiológicas y moleculares que promueven la acumulación de licopeno en los frutos de pomelo SR al madurar en ausencia de luz. En los frutos que permanecieron tapados durante el cambio de color se produjo una aceleración de la desverdización natural, un aumento de hasta 50 veces del contenido de licopeno y una notable reducción de xantofilas y ABA, metabolitos de etapas posteriores de la ruta. Estos resultados sugieren que la ausencia de luz provoca un bloqueo en la ruta de biosíntesis a nivel de la ciclación del licopeno. El estudio de la expresión de los genes de la ruta de biosíntesis de carotenoides reveló un efecto estimulador de la luz sobre la transcripción de la mayoría de los genes. En este sentido cabe destacar que los pomelos presentan una menor expresión y actividad de la enzima *βLCY2*, clave en la ciclación de licopeno en frutos cítricos, y su menor expresión en frutos tapados acentuaría de forma notable la acumulación de licopeno. Paralelamente, en los frutos tapados la ausencia de luz provocó una aceleración en la transición de cloro- a cromoplastos y un aumento en la expresión de las chaperonas *HSP20-4* y *HSP21*. Estos cambios podrían estar directamente relacionados con la diferenciación de nuevas estructuras a nivel de los plástidos que favorecerían la síntesis y acumulación de carotenoides. La aparición de numerosos cristales de licopeno en frutos tapados, no detectados en los no tapados, sugiere una estrecha relación entre la presencia de determinadas estructuras y los carotenoides que se acumulan. En la última parte de este trabajo se investigó la posible relación entre el perfil de carotenoides en la piel y la pulpa de frutos cítricos con distinta composición, y el tipo de subestructuras que se desarrollan en los plástidos, ya que estos orgánulos constituyen el sito de síntesis y acumulación de carotenoides. La composición en carotenoides parecería determinar la ultraestructura de los plástidos. Los plastoglóbulos y las membranas aclorofílicas fueron las estructuras más abundantes en los cromoplastos de la piel de los frutos de naranjas y pomelos, siendo probablemente lugares activos de síntesis y acumulación de fitoeno y β,β-xantofilas. La presencia de licopeno se asoció a estructuras cristalinas alargadas, mientras que la inusual y elevada concentración de carotenos incoloros en la pulpa del mutante amarillo Pinalate fue paralela a la presencia exclusiva de un nuevo tipo de subestructuras globulares. Los resultados demuestran que los plástidos de los frutos cítricos presentan una plasticidad extraordinaria, lo que permite una redistribución de los carotenoides en diferentes compartimentos celulares que puede potenciar su acumulación y evitar posibles efectos deletéreos.

1. INTRODUCCIÓN

1.1. Frutos Cítricos

1.1.1. Origen y principales especies comerciales

El origen de los cítricos conlleva un gran número de historias, leyendas y controversias. Aunque algunos investigadores los refieren como nativos de las zonas tropicales y subtropicales del sureste asiático (China, India y el Archipiélago Malayo) (Gmitter y Hu, 1990; Ollitrault y Navarro, 2012; Liu et al., 2012), investigaciones más recientes apuntan a que algunas especies comercializadas hoy en día podrían ser originarias de Australia (Nueva Caledonia) y Nueva Guinea, siendo lentamente introducidos en diferentes regiones del mundo (Liu et al., 2012). El consumo de cítricos data desde el año 2205 a 2197 aC, particularmente mandarinas y pumelos, que se consideraban muy valiosos y únicamente disponibles para los miembros de la corte imperial. El limón se reconoce como originario de la India y las naranjas y mandarinas, de China. Desde entonces, tanto la producción como el procesado y comercialización de cítricos no ha hecho más que crecer, situando a este cultivo como uno de los más importantes a nivel mundial (Liu et al., 2012).

Las especies más cultivadas y comercializadas actualmente a nivel mundial en orden de importancia son: naranja dulce (*Citrus sinensis* L. Osbeck), mandarinas, clementinas y sus híbridos (*C. reticulata* Blanco y *C. clementina*), así como también Satsumas (*C. unshiu*), limones (*C. limon*) y pomelos (*C. paradisi* Macf.). Recientemente se ha publicado la secuenciación del genoma de los frutos cítricos, así como también un análisis exhaustivo de la filogenia y evolución de los híbridos naturales en este género durante el proceso de domesticación (García-Lor et al., 2012; Garcia-Lor et al., 2013; Xu et al., 2013; Wu et al., 2014). Se ha sugerido que las especies primarias ancestrales son *C. medica* L. (cidro), *C. maxima* (Burn.) Osbeck (pumelo) y *C. reticulata* Blanco (mandarinas) y mediante diferentes cruzamientos y mutaciones espontáneas se han originado el resto de las especies. Por lo tanto, el genoma de la mayoría de las especies cítricas cultivadas a nivel mundial es un mosaico de distintos fragmentos de ADN de las especies ancestrales surgidas de diferentes eventos de recombinación interespecífica (García-Lor et al., 2012). Sin embargo, aún existen dudas en relación a las líneas puras o ancestrales debido a la falta de documentación y existe también controversia, principalmente en el caso de las mandarinas, donde no se ha podido encontrar una especie pura original (García-Lor et al., 2012; Wu et al., 2014). De este trabajo también surge un posible nexo filogenético entre mandarinas ancestrales (Ponkan, Willowleaf o Huanglingmiao) y naranja dulce o pumelo, debido a que comparten una proporción importante del genoma (Wu et al., 2014).

1.1.2. El pomelo (*Citrus paradisi*)

A diferencia de otros cítricos, el pomelo es originario de la zona del Caribe, de la isla de Barbados, evolucionando mayoritariamente en dicha zona desde principios del siglo XVIII (Scora et al., 1982). La especie *C. paradisi* fue descrita por primera vez por James Macfadyen en 1837, que destacó la existencia de dos tipos de frutos: por un lado los que presentaban pulpa blanca y aspecto maliforme y por otro, los de pulpa roja y piriformes (Macfadyen, 1837). A su vez, la clasificación de los pomelos como una especie diferente es muchas veces cuestionada, ya que su

origen se remonta a una hibridación entre *Citrus maxima* (pumelo) y *Citrus sinensis* (naranja dulce), por lo que sería más correcta su clasificación como un híbrido interespecífico (Gmitter, 1995; de Moraes et al., 2007). Lo anterior se ha confirmado recientemente mediante análisis de polimorfismos (SNPs), que indican la presencia de genes provenientes de *C. maxima* (70%) y de *C. reticulata* (30%), estos últimos provenientes del parental *C. sinensis* (Garcia-Lor et al., 2013). La diversidad actual en este grupo se debe principalmente a mutaciones somáticas (espontáneas o inducidas) surgidas en los últimos dos siglos (Hodgson, 1967), mientras que hibridaciones intra- e interespecíficas han resultado en nuevos cultivares híbridos interesantes y con características diferenciales, constituyéndose en una especie atractiva como parental (Gmitter, 1995).

La producción mundial de pomelos asciende a 8 millones de toneladas (FAO, 2012), siendo China el principal productor, seguido por Estados Unidos, que representa el 40% de las exportaciones de este fruto, y Méjico. Cuba e Israel, así como Sudáfrica, son también productores importantes de esta especie cítrica. Por otro lado, el 64% de la producción mundial se destina al consumo en fresco (FAO, 2012), siendo determinante su calidad cosmética como criterio de aceptabilidad. El resto se destina a la industria, principalmente a la elaboración de alimentos (FAO, 2012). En el caso de España, la producción de pomelos es más reducida que la de otros cítricos, superando las 55.000 toneladas en 2012. Sin embargo, es un fruto en donde el consumo en fresco y la exportación son claves, ya que el 95% de la producción se destina a la exportación (FAO, 2012).

1.1.3. Producción y comercialización de cítricos: importancia del consumo en fresco

Debido a su sabor característico y ampliamente aceptado por los consumidores, su accesibilidad desde el punto de vista económico y sus propiedades beneficiosas para la salud, los frutos cítricos son ampliamente consumidos a nivel mundial, conllevando un elevado impacto económico tanto en países desarrollados como en desarrollo (Liu et al., 2012). Los cítricos constituyen la principal fruta de exportación a nivel mundial, con un intercambio anual de entre 12 y 15 millones de toneladas y una producción total que ronda los 118 millones de toneladas (FAO, 2014), considerándose el frutal más importante en el mundo (Liu et al., 2012; Sidana et al., 2013). El destino principal de estos frutos (70%) es el consumo en fresco mientras que el 30% restante se destina a la industria. La naranja dulce es el cítrico más abundante a nivel mundial (58%), seguido por las mandarinas y sus híbridos (23%), limones y limas (13%) y pomelos (6%) (FAO, 2014).

España es el principal productor de mandarinas del mundo, el mayor productor de cítricos de Europa y el sexto mundial, con una producción anual de 5,5 millones de toneladas, exportando en promedio un 77% de su producción, lo que lo convierte en el mayor exportador mundial de fruta fresca (FAO, 2012-2014). Destaca principalmente la producción de naranjas (53%) y mandarinas (34%), seguidas por limones y limas (12%) y pomelos (1%). La superficie cultivada es de 330.000 ha, con un 60% localizada en la Comunidad Valenciana, la cual representa el 76% del total de cítricos exportados (GVA, 2014). El 83% de la producción en España se destina al consumo en fresco y el resto a la industria. El principal destino comercial de la fruta española es la Unión Europea (80% de las mandarinas, 70% de las naranjas y 90% de los limones) aunque también exporta a Japón y Estados Unidos (GVA, 2010; 2014).

El consumo mundial de fruta cítrica se sitúa en 24-25 kg/persona/año y en España, en 28 kg de fruta/persona/año (MARM, 2010), siendo las naranjas la especie más consumida (20 kg/persona/año), seguida por mandarinas (6.5 kg/persona/año) y limones (2.1 kg/persona/año), existiendo una clara tendencia al aumento en el consumo desde el año 2002 (MARM, 2010). El aporte de vitamina C, compuestos fenólicos (flavonoides principalmente) y carotenoides (algunos de ellos son precursores de la vitamina A), unidos a su característico sabor y facilidad de consumo (en fresco o zumo), son factores determinantes en la elección de este tipo de fruta por el consumidor (Sidana et al., 2013). Los "cítricos valencianos" constituyen una Indicación Geográfica Protegida desde el año 2000 (GVA, 2010), debiendo cumplir con ciertos requisitos de calidad de fruta (tamaño, contenido de zumo, índice de madurez), formato de envase, para ser parte de esta indicación de calidad "extra". Debido a la importancia del comercio internacional en estas frutas y la valorización de consumo en fresco gracias a las características nutricionales y saludables, es necesario contar con métodos que permitan mantener, o si es posible mejorar, la calidad y las características organolépticas del producto durante el transporte y comercialización en los diferentes mercados de destino.

1.2. La conservación postcosecha a bajas temperaturas

El almacenamiento refrigerado a bajas temperaturas (2-5 ºC) es una de las tecnologías más utilizadas comercialmente para extender la vida postcosecha de diversas frutos y hortalizas (Singh, 2011), entre los que se incluyen los cítricos (Lafuente y Zacarías, 2006). La conservación a bajas temperaturas permite reducir la respiración de los frutos y el crecimiento de potenciales patógenos, minimizando los procesos que llevan a la senescencia y el deterioro (Singh, 2011). Sin embargo, existe un gran número de especies sensibles a estas condiciones y que manifiestan distintos tipos de alteraciones y síntomas como consecuencia de diversos procesos metabólicos inducidos por el frío que, genéricamente, se conocen como daños por frío (DF) (Lyons, 1973; Lafuente et al., 2005; Sevillano et al., 2009).

Las especies sensibles a desarrollar DF presentan, por lo general, una vida postcosecha más limitada, debido a la imposibilidad de utilizar las bajas temperaturas por períodos prolongados, que permita extender su conservación y vida útil. A pesar de que existen diversos síntomas para un mismo desorden fisiológico, los cambios a nivel celular son similares entre frutos, incluyendo un hinchamiento y desorganización de las mitocondrias y los cloro- y/o cromoplastos; en concreto a nivel de los plástidos, los tilacoides se dilatan y desorganizan, perdiendo su estructura característica, y hay acumulación de micelas de lípidos en el interior de los cloroplastos (Lyons, 1973; Kratsch y Wise, 2000). El DF puede observarse durante todas las etapas de desarrollo de las plantas, pero su intensidad dependerá del tiempo y temperatura, tipo de órgano y estado de desarrollo del mismo. A su vez, la intensidad del daño que manifiestan los frutos durante su postcosecha depende no sólo de las condiciones de almacenamiento, sino también del ambiente en el cual se desarrolló el mismo (Lyons, 1973).

1.2.1. Daño por frío (DF) en los cítricos: síntomas y factores determinantes

El DF en los frutos cítricos se manifiesta con diversa sintomatología, aunque la más común es la conocida como “picado” o “pitting” en el flavedo (parte externa coloreada de la piel) de los frutos. Los DF característicos en frutos de mandarina o de pomelo se inician como picados o zonas deprimidas más o menos circulares que se oscurecen con el tiempo hacia una coloración marrón-negra, formando manchas de tamaño variable. En las naranjas, el DF se manifiesta como un manchado extendido superficialmente que se denomina “bronceado”, mientras que en el caso de los limones pueden aparecer hendiduras individuales distribuidas por la superficie del fruto y se conoce como “peteca” (Lafuente y Zacarías, 2006; Fig. 1). Los síntomas de DF, en general, se restringen al flavedo y no afectan a la pulpa, pero deprecian de forma considerable la calidad visual y comercial del fruto (Sala, 1998; Lafuente y Zacarías, 2006). La sensibilidad a los DF varía en función de la especie y variedad, siendo las limas, limones y pomelos las especies más sensibles, seguidas por mandarinas (incluyendo híbridos de éstas) y naranjas (Muñoz-Delgado, 1987; Lafuente et al., 2005). Existen, sin embargo, frutos de variedades de mandarinas sensibles (como Nova y Fortuna) o resistentes (como Hernandina y Clemenules) a los DF (Cuquerella Cayuela y Martínez-Jávega, 1995; Lafuente et al., 2005; Lafuente y Zacarías, 2006).

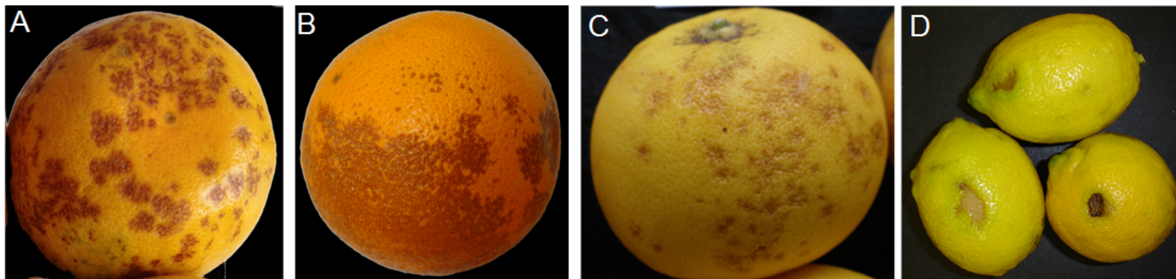

Figura 1. Síntomas característicos de daño por frío en frutos de mandarina Satsuma (A), naranja Navel (B), pomelo Marsh (C) y limón Lisbon (D).

Los frutos de pomelo se encuentran dentro de las especies cítricas más sensibles al DF, con una alta susceptibilidad a desarrollar lesiones cuando se almacenan a temperaturas inferiores a 10 ºC durante períodos extensos de tiempo y que, en general, deterioran notablemente la calidad externa (Fig. 2; Schirra, 1992; Schirra et al., 1998; Dou, 2005). En un trabajo realizado con frutos de la variedad de pomelo rojo Star Ruby, se muestra que el almacenamiento durante un mes a 4 ºC provocó la aparición de DF en la piel, mientras que los daños se redujeron significativamente a 8 ºC y no fueron detectados en frutos almacenados a 12 ºC (Schirra, 1992). Los síntomas de DF en pomelos son similares a los descritos en otros cítricos, comenzando con la aparición de pequeños picados o zonas deprimidas del flavedo (Fig. 2A) que posteriormente aumentan en número y tamaño (Fig. 2B), formando grupos o conjuntos de manchas que se ennegrecen y llegan a alcanzar extensiones de la superficie del fruto relativamente amplias, dependiendo del grado de sensibilidad y del período de conservación en frío (Fig. 2C-D). La aparición de estos síntomas se relaciona directamente con daños a nivel celular, provocando una desorganización masiva, la extrusión de lípidos fuera de los plástidos y el colapso de la membrana vacuolar o tonoplasto. El citoplasma de las células en las zonas que presentan ennegrecimiento se colapsa, se vuelve denso y vesiculado,

mientras que en el centro de las manchas no es posible discernir ningún tipo de orgánulo (Platt-Aloia y Thomson, 1976).

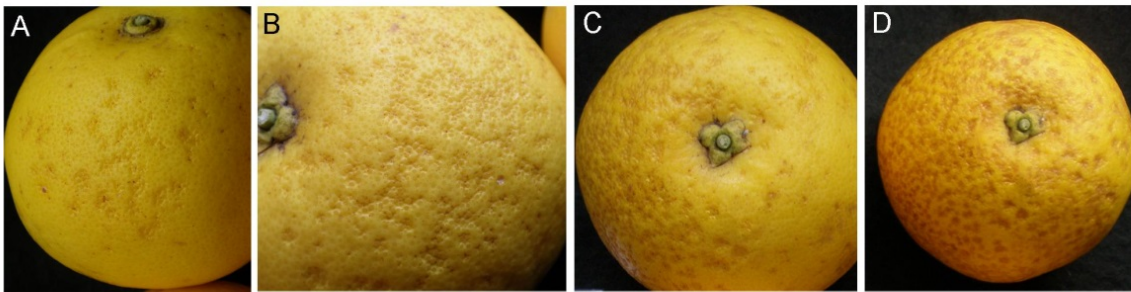

Figura 2. Evolución de los síntomas de daño por frío en frutos de pomelo Marsh almacenado durante 14 (A), 28 (B), 42 (C) y 58 días (D) a 2 ºC.

Existen distintos factores que afectan la susceptibilidad de los frutos de pomelo al DF. Así, los frutos recolectados a principio y fin de la temporada son más susceptibles al DF (Purvis, 1979; Schirra et al., 1998; Dou, 2005). Existen también diferencias en la tolerancia al DF debidas a características climáticas y de cultivo de las diferentes zonas de producción (Dou, 2005), así como variaciones de sensibilidad entre las distintas variedades de pomelo. El pomelo blanco Marsh es altamente sensible al frío, seguido por las variedades rojas Star Ruby y Red Blush (Schirra et al., 1998). A su vez, los frutos más pequeños y aquellos procedentes de la parte externa del árbol son más susceptibles a desarrollar DF (Martínez Jávega, 2002; Salvador et al., 2007). También son importantes las temperaturas durante el crecimiento y maduración de los frutos (Kawada et al., 1978; Gonzalez-Aguilar et al., 2000). El riesgo de aparición de síntomas durante el almacenamiento es mayor cuanto mayor es el tiempo de permanencia en cámara y menor la temperatura. Sin embargo, en algunas ocasiones, los síntomas no se hacen visibles mientras la fruta permanece en cámara fría, pero se manifiestan al traspasar los frutos a temperatura ambiente (Schirra, 1992). A pesar del conocimiento de la susceptibilidad de las distintas especies y variedades cítricas al DF, no siempre es posible mantener la temperatura dentro de los límites recomendados, ya sea por la necesidad de controlar el crecimiento de patógenos durante el almacenamiento, mantener la calidad de la fruta durante períodos de transporte más extensos, o incluso por imposición de tratamientos cuarentenarios por parte de los países importadores (Biolatto et al., 2005; Bassal y El-Hamahmy, 2011). Por ejemplo, para el envío de cítricos españoles a países libres de plaga de la mosca del Mediterráneo (*Ceratitis capitata*) se aplican tratamientos cuarentenarios en tránsito que consisten en aplicación de temperaturas por debajo de 2 ºC durante al menos 16 días. Para la exportación a Japón, la normativa exige temperaturas inferiores a 2 ºC en el centro de la fruta durante un período mínimo de 16 días (limones) o 17 días (naranjas y mandarinas). Para los envíos a EEUU de mandarinas y naranjas se requieren temperaturas máximas de cuarentena entre 1,1 ºC y 2,2 ºC, con períodos de permanencia de la fruta entre 14 y 18 días respectivamente (Martínez Jávega, 2002; Salvador et al., 2007).

1.2.2. Alternativas para reducir los DF en los frutos cítricos

Debido a que no siempre es posible almacenar los frutos cítricos a una temperatura que no provoque DF, la búsqueda de estrategias que permitan potenciar la resistencia de la fruta a dicho desorden fisiológico ha sido el foco de numerosos trabajos (Whitaker, 1994; Rodov et al., 1995; Sala y Lafuente, 1999; Porat et al., 2000; Rodov et al., 2000; Porat et al., 2003; Sanchez-Ballesta et al., 2003; Sapitnitskaya et al., 2006; Bassal y El-Hamahmi, 2011; Maul et al., 2011). La aplicación de altas temperaturas previamente al almacenamiento refrigerado es una de las tecnologías más evaluadas para minimizar el impacto del DF en diferentes productos vegetales (Lurie, 1998; Martínez Jávega, 2002). En el caso de los frutos cítricos, el acondicionamiento a temperaturas intermedias (16-21 ºC) durante 3 a 7 días de forma previa al almacenamiento refrigerado surge como una alternativa para minimizar el DF en frutos de diversas variedades, entre los que se encuentran los pomelos Marsh y Star Ruby (Lafuente et al., 2005; Sapitnitskaya et al., 2006; Maul et al., 2011). Este tratamiento también fue efectivo reduciendo el DF en el híbrido Oroblanco (*C. grandis* x *C. paradisi*) y para mantener la calidad sensorial durante el almacenamiento refrigerado (Porat et al., 2003). Resultados similares se obtuvieron en limones pre-acondicionados durante 1 semana a 15 ºC (Obenland et al., 1997). El acondicionamiento a temperaturas medias provoca cambios a nivel transcripcional de genes de diferentes rutas metabólicas, destacando genes de respuesta a estrés y defensa antioxidante, así como el metabolismo lipídico, implicados en la adaptación de los frutos al posterior estrés por frío (Sapitnitskaya et al., 2006; Maul et al., 2011). El acondicionamiento térmico en pomelos Marsh redujo el DF y esto estuvo asociado a una menor expresión de la ACC oxidasa (*ACO*), enzima clave en la síntesis de etileno, y de otros genes tales como la galactinol sintasa, oxigenasa o lipocalina, cuya expresión fue mayor en frutos no acondicionados (Maul et al., 2011). Por otro lado, el acondicionamiento favoreció el aumento en la expresión de genes de la enzima catalasa, una proteína de transferencia de lípidos y una proteína de dedos de zinc (zinc-finger protein) de respuesta a estrés, vinculados con la resistencia a este desorden (Maul et al., 2011). Por lo tanto, la mayor tolerancia al DF en frutos acondicionados parece deberse a la modificación de diferentes rutas metabólicas que actúan en concierto y de forma coordinada, que se pueden activar no sólo por el tratamiento de calor sino por la sinergia del mismo con la exposición posterior al frío.

Otro tratamiento efectivo de acondicionamiento térmico, previo a la conservación refrigerada, es el denominado como “curado”, en el que los frutos se someten a mayor temperatura (36-37 ºC) durante 1 a 3 días y que han demostrado ser altamente efectivos reduciendo el DF en numerosas especies de cítricos, como la mandarina Fortuna (Sala et al., 1999; Sanchez-Ballesta et al., 2003; Holland et al., 2012) o los pomelos (Porat et al., 2000). Estos tratamientos minimizan los cambios a nivel de la pared celular y el contenido de pectinas en la piel de los frutos, favoreciendo la formación de puentes de calcio, mientras que no se observaron cambios en otros componentes claves (celulosa y hemicelulosa) de la pared (Holland et al., 2012). A su vez, el curado indujo la expresión de genes relacionados con la tolerancia al estrés oxidativo (Sanchez-Ballesta et al., 2003), principalmente del sistema antioxidante, como es el caso de las enzimas catalasa, ascorbato peroxidasa y superóxido dismutasa (Sala y Lafuente, 1999). Otros metabolitos como las poliaminas (putrescina, esperimidina, espermina) podrían tener un papel protector frente al DF, ya que en

mandarina Fortuna se ha descrito una aumento (principalmente en espermina y espermidina) como respuesta al curado a 37 ºC (Gonzalez-Aguilar et al., 2000; Lafuente et al., 2005). De forma similar, tratamientos de calor intermitente (ej. ciclos de 3 semanas a 3 ºC + 3 semanas a 15 ºC durante 25 semanas o ciclos de 3 semanas a 2 ºC + 1 semana a 11 ºC) han sido propuestos como otra alternativa para minimizar el DF en frutos cítricos (Davis y Hofmann, 1973; Schirra y Cohen, 1999; Porat et al., 2003). Otros tratamientos similares (ciclos de 2 semanas a 3,5 ºC + 1 semana a 19,5 ºC durante 60 y 70 días) han resultado efectivos para evitar el DF en mandarinas Nagpur (Ladaniya, 2011).

Más recientemente se han descrito otras alternativas como el lavado a altas temperaturas (por ejemplo 53 ºC durante 3 min o 60-62 ºC durante 20-30 segundos) para minimizar el DF en frutos de pomelos, y se ha comprobado un efecto sinérgico al combinarlo con tratamientos de pre-acondicionamiento a temperaturas medias (Porat et al., 2000; Sapitnitskaya et al., 2006). Resultados similares se obtuvieron en limón Eureka, pomelo Marsh y el híbrido Oroblanco, en los que el tratamiento con agua caliente a 53 ºC durante 2-3 minutos redujo de forma importante la incidencia de DF (Rodov et al., 1995), así como también en naranjas Navel y Valencia (Bassal y El- Hamahmi, 2011). En su conjunto estos tratamientos, basados en un aumento breve y brusco de la temperatura, provocan cambios en la expresión de genes relacionados con la repuesta al frío, principalmente la expresión de enzimas antioxidantes, proteínas de choque térmico y dehidrinas (proteínas de protección frente a estrés hídrico) (Porat et al., 2002; Sapitnitskaya et al., 2006). Así, se ha comprobado que en los frutos de pomelo el tratamiento con agua caliente mantuvo constante la expresión de un gen que codifica para una dehidrina (*cor15*), mientras que en los frutos no tratados térmicamente la expresión de la misma se redujo durante el almacenamiento, sugiriendo que las dehidrinas formarían parte del proceso de inducción de tolerancia al DF en pomelos (Porat et al., 2002). En el mismo sentido, la aplicación de agua caliente a 62 ºC aumentó la expresión de distintos genes que codifican para proteínas de choque térmico (HSPs), que no se indujeron por otros estreses (radiación UV, anaerobiosis, heridas) o por la aplicación de etileno exógeno, sugiriendo que estas proteínas ejercerían un papel general en la protección de las células frente al estrés por altas y bajas temperaturas (Rozenzvieg et al., 2004).

Se han descrito otras alternativas químicas en la reducción de los DF, como es el caso del fungicida tiabendazol (TBZ) o la aplicación de ceras y diferentes tipos de coberturas (Schirra et al., 2000; Dou, 2004; Kellerman et al., 2014). La aplicación de TBZ por inmersión y con cera redujo la incidencia de DF en naranjas Valencia y en clementinas almacenadas durante 40 días a -0.5 ºC, mientras que el efecto no fue tan marcado en mandarina Satsuma (Kellerman et al., 2014). De forma similar, la aplicación de este principio activo mediante inmersión redujo la incidencia de DF en frutos de pomelo Star Ruby conservados 6 semanas a 2 ºC, siendo el efecto más pronunciado cuanto más avanzada era la fecha de cosecha (Schirra et al., 2000). La aplicación de ceras también redujo la incidencia de DF en frutos de pomelo Marsh conservados 2 meses a 4 ºC, siendo más efectiva la carnauba que el polietileno (Dou, 2004). La aplicación postcosecha de aceites esenciales de cártamo en combinación con agua (20%) en frutos de pomelo Redblush provocó una reducción importante del DF después de más de tres meses a 3 ºC (Aljuburi y Huff, 1984).

Sin embargo, y a pesar de contar con diversos tratamientos efectivos para minimizar el impacto del DF en frutos cítricos, a nivel comercial aún no se dispone de alternativas para el control de este desorden fisiológico, ya que las temperaturas efectivas para la inducción de resistencia están muy próximas a las temperaturas que pueden provocar fitotoxicidad y daños en los frutos, dependiendo de la sensibilidad varietal (Ghasemnezhad et al., 2008).

1.2.3. Papel del etileno en el DF en los frutos cítricos

El etileno (C_2H_4) es una fitohormona que afecta a diferentes etapas de crecimiento y desarrollo de las plantas, incluyendo la maduración y senescencia, así como en la respuesta a distintos tipos de estrés biótico y abiótico en las plantas (McManus, 2012; Wang et al., 2013; Rodrigues et al., 2014). La biosíntesis del etileno en las plantas superiores está bien caracterizada y se puede resumir de la siguiente forma. El etileno se origina a partir del aminoácido metionina, que se convierte en el intermediario S-adenosil-metionina (SAM), por la acción de la enzima SAM sintasa. Posteriormente, el SAM se convierte en ácido 1-aminociclopropano-1-carboxílico (ACC) mediante la acción de la enzima ACC-sintasa (ACS), que es la primera enzima específica de la producción de etileno, y el ACC es convertido en etileno mediante la acción de la enzima ACC-oxidasa (ACO) (Fig. 3). La ACO requiere la presencia de oxígeno para su actividad, y es una dioxigenasa dependiente de ascorbato y Fe que se estimula por bajas concentraciones de CO_2 (McManus, 2012). La biosíntesis de ACC, por la ACS es el paso clave limitante en la síntesis de etileno, mientras que la actividad de la enzima ACO es modulada en diferentes situaciones del desarrollo y del estrés, pero en la mayoría de casos no es una etapa limitante para la producción de etileno.

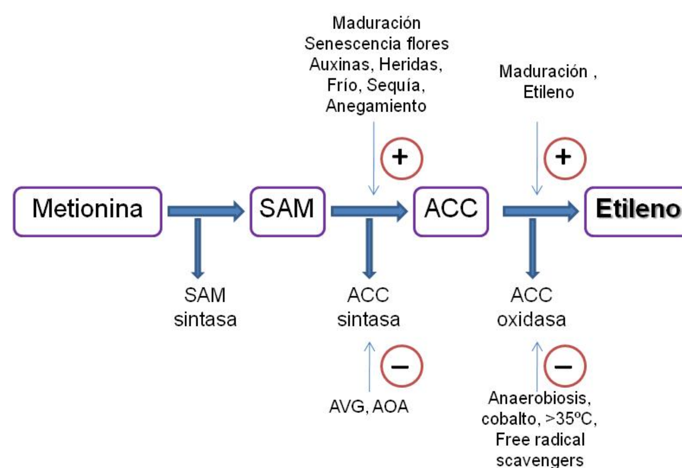

Figura 3. Esquema representativo de la biosíntesis del etileno en las plantas y los factores que la promueven (+) o inhiben (-). ACC (ácido carboxílico-1-aminociclopropano), SAM (S-adenosin-metionina).

Finalmente, la percepción, señalización y transducción del etileno en las plantas es posiblemente la mejor caracterizada entre las distintas hormonas vegetales, y se conoce con bastante precisión las diferentes familias de los receptores celulares que unen etileno, elementos que interactúan con los receptores y traducen la señal, desencadenando los cambios en la expresión

de genes que regulan o intervienen en las respuestas fisiológicas al etileno (Giovannoni, 2007; McManus, 2012; Wang et al., 2013; Gapper et al., 2013; Lacey y Binder, 2014; Rodrigues et al., 2014). En el caso del etileno, el concepto que se ha desarrollado es que en ausencia de la hormona los receptores (tipo ETR y ERS) están físicamente interactuando con otras proteínas (CTR) que mantienen reprimida la ruta de señalización. La unión del etileno a su receptor hace que se pierda la unión con las proteínas CTR y se deja de reprimir la ruta de señalización, activándose una cascada de señales y de actividades quinasa que finalmente conducen a la expresión de factores de transcripción que activan o reprimen genes de respuesta al etileno (Wang et al., 2013; Gapper et al., 2013; Fig. 4).

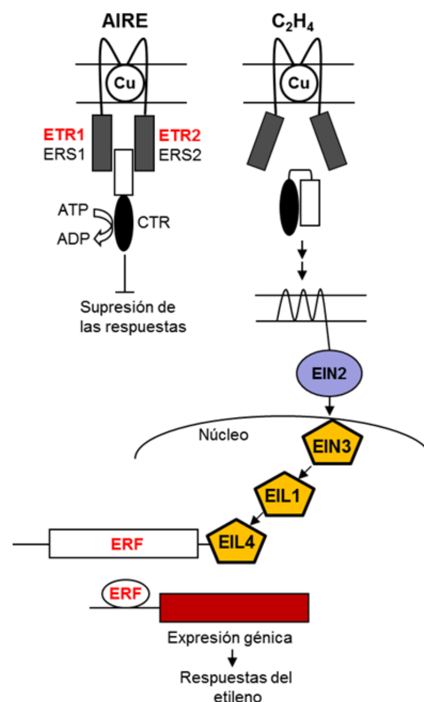

Figura 4. Esquema representativo de la percepción y señalización del etileno en las plantas. ETR/ERS-receptores de etileno; CTR-triple respuesta constitutiva-MAP-triple-quinasa; EIN2 (ethylene insensitive 2)- regulador positivo de respuesta a etileno; EIN3-EIL4-factores de transcripción reguladores de respuesta a etileno; ERF-factores de respuesta a etileno.

Las tecnologías modernas de conservación de frutas y hortalizas apuntan en su mayoría a minimizar la producción y acción del etileno durante la cosecha, el almacenamiento y transporte (Blankenship y Dole, 2003; Watkins, 2006; Martínez-Romero et al., 2007). La utilización del antagonista de la acción del etileno, 1-metilciclopropeno (1-MCP), que se une irreversiblemente a sus receptores, es una de las tecnologías que ha resultado más exitosas en los últimos años en la postcosecha de frutas y hortalizas (Blankenship y Dole, 2003; Watkins, 2006; Martínez-Romero et al., 2007). A presión y temperatura estándar, el 1-MCP es un gas, con un peso molecular de 54 g/mol y una estructura química (C_4H_6) similar a la del etileno, siendo capaz de ocupar los receptores del mismo, y evitar su unión y por lo tanto, las posteriores respuestas metabólicas que se desencadenan (Blankenship y Dole, 2003). Su afinidad por estos receptores es 10 veces mayor que

la del etileno, siendo activo incluso a menores concentraciones (Sisler y Serek, 1997). El 1-MCP se utiliza actualmente en el manejo postcosecha de numerosos frutos climatéricos (manzanas, aguacate, mango, caqui, entre otros), siendo la manzana, el fruto en el que se ha registrado mayor aplicación comercial (Watkins, 2006). Posee diversas ventajas entre las que se citan su facilidad de aplicación, su mecanismo de acción no tóxico, que no deja residuos sobre el producto tratado y que es activo a muy bajas concentraciones (Watkins, 2006). En el caso de los cítricos, su uso no es extendido, debido a que sus efectos sobre la maduración no son tan marcados y que, a su vez, provoca una mayor susceptibilidad al ataque de patógenos (Porat et al., 1999; Marcos et al., 2005). Sin embargo, el 1-MCP es una herramienta muy valiosa a la hora de estudiar el papel del etileno en distintos procesos metabólicos, entre los que se incluye el DF.

Los frutos cítricos exhiben una baja tasa respiratoria y de producción de etileno, por lo que se clasifican como frutos no climatéricos (Eaks, 1960; Baldwin, 1993; Cherian et al., 2014). Sin embargo, a pesar de producir muy bajos niveles de la hormona y de no presentar la respuesta auto-catalítica característica de frutos climatéricos, los cítricos son capaces de responder a la aplicación exógena de la hormona, la cual provoca diversos cambios en el metabolismo. La respuesta más clara al etileno ocurre durante el proceso de desverdizado de los frutos cítricos, en donde su aplicación exógena estimula la degradación de clorofilas en la piel junto con la acumulación de carotenoides coloreados, lo que acelera la coloración externa del fruto (Goldschmidt et al., 1993; Porat, 2008). Se ha demostrado en frutos cítricos que el almacenamiento en frío estimula la producción de etileno y que dicho aumento se relaciona con la aparición de DF aunque aún se desconoce si el aumento en la producción de la hormona es causa o consecuencia del daño (McCollum y McDonald, 1991; Lafuente et al., 2003; Sanchez-Ballesta et al., 2003; Zacarías et al., 2003).

Algunos trabajos apuntan a la hipótesis del etileno como promotor del DF en especies cítricas sensibles, basados en la observación de que la aplicación de la hormona previa el almacenamiento refrigerado provoca un aumento de los DF (Yuen et al., 1995; Lafuente et al., 2001; Gosalbes et al., 2004). A su vez, la reducción de la temperatura estimula la síntesis de ACC, seguido por una estimulación de la actividad de la ACO (Zacarías et al., 2003), así como favorece la transcripción de genes que codifican para las enzimas de biosíntesis de la hormona (Maul et al., 2008). Sin embargo, otros estudios indican un posible papel protector o de defensa del etileno, ya que la aplicación exógena continua a bajas concentraciones durante el almacenamiento refrigerado reduce la incidencia de DF (Lafuente et al., 2004) o, por otro lado, la inhibición de su percepción (mediante la aplicación del 1-MCP) favorece la aparición de DF en naranjas y mandarinas sensibles (Porat et al., 1999; Lafuente et al., 2001). La inhibición de la percepción del etileno no siempre redunda en un aumento en la incidencia del DF, efecto que depende de la variedad estudiada, ya que en mandarinas Nova y Murcott y en el tangor Ortanique, la aplicación de 1-MCP redujo los síntomas de DF (Salvador et al., 2006; Edagi et al., 2010). Por lo tanto, las respuestas desencadenadas por los DF en los frutos cítricos son muy diversas y complejas, afectando a diferentes procesos metabólicos y el papel del etileno en este proceso aún no se ha dilucidado completamente.

En relación a la biosíntesis de la hormona en frutos cítricos, se han identificado dos genes que codifican para la enzima ACS y uno para la enzima ACO (Mullins et al., 2000) que se expresan diferencialmente en respuesta a diversos estreses bióticos (Marcos et al., 2005) o abióticos (Wong et al., 1999; Maul et al., 2008), respondiendo a un control auto-inhibitorio (Marcos et al., 2005). En general, los genes de biosíntesis de etileno se inducen como respuesta al frío en los frutos cítricos, favoreciendo la síntesis de la hormona durante el almacenamiento refrigerado. Se ha observado que al trasferir los frutos desde las bajas temperaturas a temperaturas óptimas para la actividad, se estimulan las enzimas ACS y ACO y aumenta sensiblemente la producción de la hormona en los frutos con síntomas de DF (Zacarías et al., 2003; Lafuente y Zacarías, 2006).

La cascada de señalización del etileno en las plantas está compuesta por una compleja red de diferentes pasos estrechamente interrelacionados (Wang et al., 2013; Gapper et al., 2013; Rodrigues et al., 2014; Cherian et al., 2014), en donde el primer mecanismo responsable de percibir la hormona se ejerce por los receptores de etileno (ETRs o ERS), una compleja familia de isoformas que varían en número en las distintas especies vegetales. Se han descrito cinco isoformas de ETRs en *Arabidopsis*, mientras que parecen existir seis elementos diferentes en tomate (Binder, 2008; Lacey y Binder, 2014). En los frutos cítricos se han identificado tres isoformas que codifican para ETRs (Katz et al., 2004; Distefano et al., 2009; John-Karuppiah y Burns, 2010; Alós et al., 2014), alguna de ellas (*ETR2*) se induce específicamente por la aplicación de etileno exógeno durante el cambio de color (Alós et al., 2014). También se ha sugerido una relación positiva entre el nivel de transcritos de receptores de etileno y la sensibilidad a la hormona en frutos cítricos (Alós et al., 2014). Por lo tanto, los cambios en la expresión de los receptores pueden ser relevantes en determinar la sensibilidad a la hormona, ya que los mismos ejercen un control negativo sobre las respuestas al etileno (Wang et al., 2013; Gapper et al., 2013).

Continuando con la cadena de señalización, los factores de respuesta al etileno o ethylene response factors (ERFs) han sido también descritos como reguladores negativos de la señal de la hormona, con un papel esencial en la maduración y respuesta a estrés de los frutos (Wang et al., 2013; Gapper et al., 2013). Existen pocos trabajos que estudien el comportamiento de estos ERFs en frutos cítricos, siendo regulados en general negativamente por el etileno (Alós et al., 2014). Otro grupo de ERFs (C-repeated binding factors-CBFs) se inducen en respuesta al estrés por frío y presentan una papel importante en la aclimatación en *Poncirus* (Champ et al., 2007). En otros frutos no climatéricos y también sensibles al frío, como el níspero (*Eriobotrya japónica* Lindl.), se ha detectado un aumento en la expresión de los genes de señalización de etileno en respuesta al frío (Wang et al., 2010), sin embargo, aún se desconoce el papel de los mismos y de los factores de respuesta al etileno en los procesos relacionados con el daño por frío en los frutos cítricos.

1.2.4. Causas y consecuencias del DF: implicación del sistema antioxidante

Existen diversas hipótesis sobre las causas que pueden generar los DF en frutos y hortalizas. Uno de los eventos primarios sería la modificación de la permeabilidad de las membranas debido a la saturación de los ácidos grasos, principalmente de las membranas mitocondriales y los plástidos (Lyons, 1973; Wismer et al., 1998; Kratsch y Wise, 2000; Sevillano

et al., 2009). A bajas temperaturas se produce la peroxidación de los ácidos grasos de membrana, así como un aumento en la relación esteroles/fosfolípidos que conlleva una reducción de la fluidez y funcionalidad de la misma (Lyons, 1973; Sevillano et al., 2009; Aghdam y Bodbodak, 2014). La evolución del daño a nivel de la membrana puede seguirse por la pérdida de electrolitos o por la acumulación de un producto final de la peroxidación de los ácidos grasos, el malondialdehido (MDA). Por lo tanto, la cantidad de MDA en el tejido es utilizada como marcador de estrés oxidativo y es un indicador de la pérdida de integridad de las membranas celulares (Hodges et al., 1999).

Se han descrito alteraciones en las enzimas integrales de las membranas o implicadas en la generación de energía y en la síntesis de proteínas en respuesta al frío. La enzima fenilalanina amonio liasa (PAL), que es el primer paso clave en la síntesis de fenil propanoides, parece jugar un papel importante en la regulación de la tasa de sustratos para la síntesis de polifenoles en respuesta al daño por frío. En la mandarina Fortuna se ha observado un aumento en la expresión de este gen y de su actividad enzimática de forma paralela a la aparición de DF (Martínez-Téllez y Lafuente, 1997; Lafuente et al., 2001). Se ha sugerido también un posible papel protector de esta enzima en el desarrollo del DF, aunque el aumento en la actividad de la misma previo al almacenamiento en frío no incrementó la tolerancia al desorden (Lafuente et al., 2001). De forma similar, la expresión del gen β-1,3-glucanasa y de una oxigenasa (*CIOX*) aumentó en forma previa y paralela al DF, únicamente en genotipos sensibles (mandarina Fortuna) y no en los tolerantes (clementina Hernandina), por lo que podrían corresponder a señales o marcadores relacionados directamente con el daño celular, además de estimularse la expresión de los genes correspondientes por la aplicación exógena de etileno (Gosalbes et al., 2004; Sanchez-Ballesta et al., 2006).

Por otro lado, las bajas temperaturas y los DF se han relacionado con la inducción de estrés oxidativo en distintos frutos (Sevillano et al., 2009; Aghdam y Bodbodak, 2014) incluyendo los cítricos (Sala, 1998; Sala y Lafuente, 2000, Sanchez-Ballesta et al., 2003). El daño a nivel celular ocurre cuando la producción de especies reactivas del oxígeno (ROS, del inglés `reactive oxygen species´: peróxido de hidrógeno-H_2O_2, superóxido-O_2^-, hidroxilo-OH^-, singlete oxígeno-$^1O_2\cdot$) excede la capacidad de las células para eliminarlas (Hodges et al., 2004), quebrándose el balance entre ambos sistemas. Dichas ROS provocan cambios a nivel de lípidos de membrana, oxidación de proteínas, inhibición de actividades enzimáticas y daños en los ácidos nucleicos, entre otros efectos. La respuesta de la célula a la agresión oxidativa, fenómeno conocido como estrés oxidativo, implica no sólo cambios cinéticos y moleculares a nivel de enzimas, co-sustratos, cofactores y moléculas antioxidantes, sino también importantes modificaciones en el perfil transcripcional y en la acumulación de distintas proteínas (Hossain et al., 2012).

Una de las respuestas más significativas durante el estrés oxidativo consiste en un aumento en la actividad de enzimas antioxidantes, así como también en la activación de los ciclos ascorbato/glutatión, el sistema de la glutatión peroxidasa y el sistema de tiorredoxinas (Møller, 2001; Fig. 5). Las actividades enzimáticas antioxidantes más representativas en las células vegetales son: catalasa (CAT), superóxido dismutasa (SOD), ascorbato peroxidasa (APX), glutatión reductasa (GR), glutatión peroxidasa (GPX), monodehidroascorbato reductasa (MDHAR)

y dehidroascorbato reductasa (DHAR) (Møller, 2001; Hossain et al., 2012). La SOD constituye la primera línea de defensa contra radicales superóxido, catalizando la dismutación del O_2, acumulando H_2O_2, que a su vez es eliminado posteriormente por la acción de la APX y CAT (Edreva, 2005). A su vez, la dismutación del H_2O_2 requiere poder reductor otorgado por el ácido ascórbico (AsA), papel que también cumple el glutatión (GSH) durante el proceso de conversión de dehidroascorbico (DHA) en AsA, con la subsecuente formación de glutatión oxidado o GSSG (Pignocchi y Foyer, 2003; Foyer y Noctor, 2005). Por lo tanto, la defensa antioxidante de las plantas está en gran parte compuesta por dos sistemas: el enzimático y el no enzimático, caracterizado este último por distintos metabolitos que otorgan poder reductor y también son capaces por si mismos de inhibir la acción de ROS, como es el caso del AsA y el GSH, componentes claves del ciclo ascorbato-glutatión (Noctor y Foyer, 1998; Hossain et al., 2012). A su vez, también existe una línea de defensa frente al estrés oxidativo relacionada con la reparación del daño provocado por las ROS, controlado principalmente por la glutatión peroxidasa (Møller, 2001).

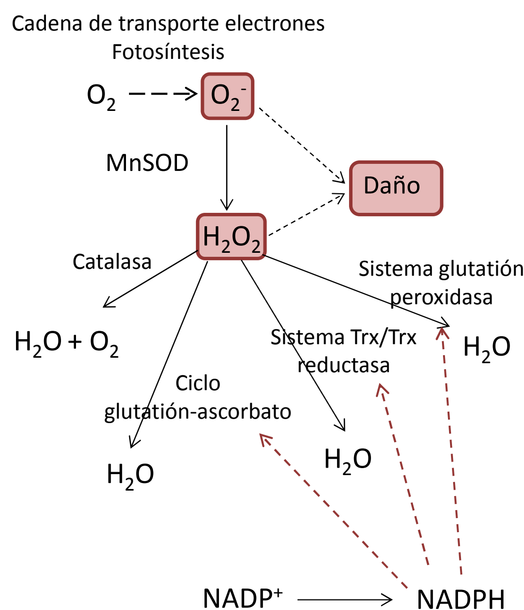

Figura 5. Esquema representativo de los principales mecanismos de respuesta antioxidante en las células vegetales. Adaptado de Moller (2001).

El aumento en la actividad de distintas enzimas antioxidantes se ha asociado a la respuesta a las bajas temperaturas y al DF en distintos frutos sensibles (Sala, 1998; Zhang et al., 2011; Yang et al., 2013; Chongchatuporn et al., 2013). Además, la aparición de DF también se ha relacionado con la acumulación de las especies reactivas superóxido y peróxido de hidrógeno (Yang et al., 2013). En distintas especies cítricas se ha comprobado un incremento en el contenido de peróxido de hidrógeno y de MDA en respuesta a las bajas temperaturas (Santini et al., 2013). De forma similar, el aumento en la actividad de la catalasa en frutos cítricos se ha relacionado con una mayor resistencia al DF, mientras que el resto de las enzimas no parecen estar involucradas directamente en la tolerancia al DF (Sala y Lafuente, 1999; Sala y Lafuente, 2000). Sin embargo, también se ha descrito que la inducción de los genes *CAT* y *SOD* podría actuar como un mecanismo de protección

frente al DF que se induce durante el pre-acondicionamiento a altas temperaturas (Sanchez-Ballesta et al., 2003; Sapitnitskaya et al., 2006; Maul et al., 2011). El posible papel del sistema antioxidante en la resistencia al DF inducida por los tratamientos con altas temperaturas también se ha sugerido en frutos de la mandarina Fortuna (Sanchez-Ballesta et al., 2003) y naranjas Navel y Valencia (Bassal y El-Hamahmy, 2011).

La capacidad antioxidante total de las células está relacionada con la habilidad de un tejido para controlar o sobrellevar el estrés oxidativo. Las técnicas desarrolladas para evaluar la capacidad antioxidante total de las muestras biológicas valoran la habilidad de los compuestos antioxidantes (donantes de un hidrógeno o un electrón) presentes en la célula, para reducir las especies oxidantes, por lo que son, en general, clasificados como métodos de inhibición directos o indirectos del poder oxidante de una molécula estándar (Huang et al., 2005). Por otra parte, otros ensayos se basan en la cuantificación de los productos formados tras el proceso oxidativo. La mayoría de los métodos de determinación de la actividad antioxidante no utilizan especies radicales que existen en la naturaleza, sino radicales que son oxidantes iniciadores ajenos al metabolismo celular como el dihidrocloruro 2',2'-azobis-2-methylpropionamida (AAPH·), el ácido 2,2’-azinobis (3-etilbenzotiazolín)-6-sulfónico (ABTS·) o el 2,2-difenil-1-picrilhidrazilo (DPPH·). Por otro lado, el empleo de los radicales peroxilo o hidroxilo en los ensayos les añade un mayor significado biológico, ya que estas especies reactivas son las más importantes a nivel fisiológico. Este tipo de ensayos son conocidos como capacidad total de secuestro de oxirradicales, capacidad de absorción de radicales de oxígeno, potencial total antioxidante de secuestro de radicales y capacidad de secuestro del oxígeno singlete (TOSC, ORAC, TRAP, SOAC por sus siglas en inglés, respectivamente) (Huang et al., 2005; Prior et al., 2005; Ouchi et al., 2010).

1.3.Carotenoides

Los carotenoides constituyen una amplia familia de compuestos isoprenoides, con más de 700 estructuras diferentes. Los carotenoides derivan de la modificación de la estructura básica del tetraterpeno lineal fitoeno (C_{40}), un caroteno incoloro que constituye el primer carotenoide en la ruta de biosíntesis (Britton, 1995a; Walter y Strack, 2011). Modificaciones sucesivas de esta estructura basal generan distintas moléculas con diversas propiedades físicas y químicas, responsables de las coloraciones amarillas, naranjas y rojas de la numerosas flores, frutos u otros órganos vegetales (Fraser y Bramley, 2004; Fig. 6). Estos compuestos también presentan una gran relevancia para la nutrición y la alimentación humana, y la industria correspondiente, debido a su capacidad antioxidante y como precursores de la vitamina A, además de su utilización como colorantes en alimentos y suplementos nutricionales (Fraser y Bramley, 2004).

1.3.1. Estructura, clasificación y propiedades de los carotenoides

Los carotenoides son tetraterpenos, compuestos por ocho unidades de isopreno formando una cadena poliénica de naturaleza lipofílica. Dicha cadena conforma el cromóforo de la molécula, que contiene entre 3 y 11 dobles enlaces conjugados que influyen directamente en sus propiedades químicas y físicas (Britton, 1995b). El número de dobles enlaces conjugados determina el cromóforo y el espectro de absorción de la molécula y, por lo tanto, su coloración. Los

carotenoides se dividen en dos grandes grupos: los hidrocarbonados (carotenos) y los oxigenados (xantofilas, Fig. 6). El esqueleto básico carbonado que compone la molécula puede ciclarse en los dos extremos, además de incorporar grupos que contienen oxígeno, formándose las xantofilas. En estos procesos actúan distintas enzimas hidroxilasas, ciclasas, ketolasas, desaturasas que conforman la ruta de biosíntesis de estos compuestos en las plantas (Fraser y Bramley, 2004). Paralelamente también pueden ocurrir modificaciones de la conformación *cis-trans*, del tamaño de la cadena o la degradación de estos compuestos en otros de menor tamaño, denominados apocarotenoides (Britton, 1995a). Los carotenoides más abundantes en las plantas contienen dos anillos β-ionona en los extremos de la molécula, entre los que destacan el β-caroteno y las β,β-xantofilas (zeaxantina, violaxantina, luteína, Fig. 6).

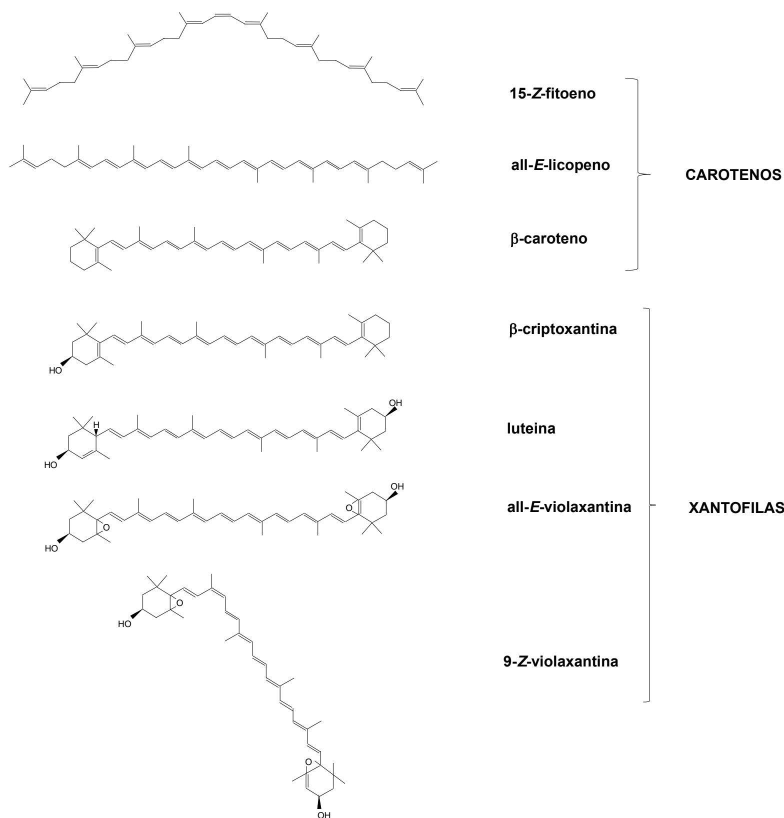

Figura 6. Estructura de los principales carotenoides presentes en los frutos cítricos.

Los carotenoides se sintetizan en organismos fotosintéticos, algunas bacterias y hongos no fotosintéticos, mientras que los animales deben obtenerlos a partir de la dieta (Fraser y Bramley, 2004). Existe, sin embargo, dos excepciones en el reino animal: el áfido del guisante (*Acyrthosiphon pisum*) y un ácaro (*Tetranychus urticae*), los cuales parecen haber adquirido la capacidad para sintetizar estos compuestos por transferencia horizontal a partir de un hongo (Moran y Jarvik, 2010; Altincicek et al., 2012). La ingesta de carotenoides en la dieta es fundamental para los animales ya que cumplen un papel importante en numerosos procesos biológicos y además pueden reportar ciertos beneficios para la salud. Los carotenoides con un anillo β-ionona no sustituido (α- y β-caroteno, β-criptoxantina) son los precursores de la vitamina A (Fraser y Bramley, 2004; Turner et al., 2011). A su vez, los carotenoides tienen una importante capacidad antioxidante y pueden actuar como protectores frente a diversas enfermedades crónicas y

degenerativas, como por ejemplo enfermedades cardiovasculares, neurológicas, diferentes tipos de cáncer así como también patologías óseas, de foto-sensibilidad de la piel o maculares (Fiedor y Burda, 2014; Berman et al., 2014). A su vez, algunos carotenoides, como es el caso del licopeno, poseen la capacidad de influir directamente en la señalización redox a nivel celular (Palozza et al., 2012).

1.3.2. Principales funciones de los carotenoides en las plantas

El número de dobles enlaces conjugados determina el cromóforo de los carotenoides y por tanto, su espectro de absorción, así como en algunas de sus funciones biológicas en las plantas. Los carotenoides presentes en los complejos antena de los cloroplastos captan energía a longitudes de onda específica y poseen la capacidad de transferirla a la clorofila, lo que permite un mejor aprovechamiento de la energía lumínica durante la fotosíntesis. A su vez, los carotenoides son capaces de disipar el exceso de energía y proteger los fotosistemas de los daños que se pueden ocasionar, función conocida como foto-protección, y que involucra directamente al ciclo de las xantofilas (Demmig-Adams et al., 1996). Los carotenoides presentan también un papel relevante como estabilizadores de las membranas celulares, principalmente en los plástidos (Havaux, 1998; Vishnevetsky et al., 1999; Cazzonelli y Pogson, 2010). Los carotenoides pueden, además, foto-proteger los fosfolípidos que componen la bicapa lipídica de las membranas de los tilacoides (Havaux, 1998). Por lo tanto, cambios en la presencia de determinados tipos de carotenoides (principalmente xantofilas y β-caroteno) pueden afectar a la fluidez de las membranas e incidir en la peroxidación de los lípidos que conforman las mismas (Havaux, 1998).

Los carotenoides también presentan una elevada capacidad antioxidante y de protección frente a los daños que generan las especies reactivas del oxígeno (ROS), que se producen en las células durante la fotosíntesis, la respiración, o ante distintos tipos de estreses (Hossain et al., 2012). El O_2 posee una estructura química que lo convierte fácilmente en captador de electrones, formando ROS como subproductos de diferentes reacciones metabólicas. Las ROS pueden provocar daños importantes en las células, afectando a la estructura de proteínas, lípidos, carbohidratos y ADN, lo que se conoce como daño oxidativo (Gill y Tuteja, 2010). El daño oxidativo en lípidos, proteínas y ácidos nucleicos se manifiesta cuando la concentración de ROS excede a la capacidad de la célula de eliminarlas, como ocurre por ejemplo cuando se someten las plantas a un exceso de luz (Edreva, 2005). El comportamiento antioxidante de los carotenoides depende estrechamente de su concentración y localización, así como de su estructura química (Edreva, 2005). Así, la capacidad antioxidante de los carotenoides está determinada por el número de dobles enlaces conjugados que posee, siendo mayor en el caso del licopeno (11), seguido por el β-caroteno (9 más dos en los anillo β), luteína y α-caroteno (9 más uno en el anillo β) y las xantofilas violaxantina y neoxantina (9) (Di Mascio et al., 1989; Meléndez-Martínez et al., 2007). En este sentido, se ha demostrado que el licopeno es el caroteno más eficiente en la inactivación del oxígeno singlete a nivel celular, presentando una elevada capacidad antioxidante (Di Mascio et al., 1989; Aizawa et al., 2011).

Finalmente, la concentración y composición de los distintos carotenoides en diversos órganos vegetales, especialmente flores y frutos, les proporciona su coloración característica y determina su función como atrayentes de polinizadores naturales o dispersores de semillas, favoreciendo así la fecundación, reproducción y dispersión (Walter y Strack, 2011).

1.3.3. Biosíntesis y acumulación de carotenoides en las plantas

1.3.3.1. Síntesis de precursores

La acumulación de carotenoides en las plantas es el resultado de un estrecho balance entre la biosíntesis, degradación y acumulación en estructuras especializadas (Li y Yuan, 2013). La biosíntesis de carotenoides tiene lugar en los plástidos, ya sea cloroplastos de tejidos verdes (fotosintéticos), o cromoplastos en los órganos o tejidos coloreados (flores y frutos, principalmente). Como isoprenoides, derivan de la unidad fundamental de 5 átomos de carbono, isopentil difosfato (IPP) y de su isómero dimetilalil difosfato (DMAPP), que pueden inter-convertirse por acción de la enzima IPP/DMAPP isomerasa (IDI). Ambos precursores se sintetizan a través de dos rutas compartimentalizadas: la ruta del ácido mevalónico (MVA), que ocurre en el citosol y la ruta del metil-eritritol-fosfato (MEP), que tiene lugar en los plástidos (Rodríguez-Concepción, 2010; Hemmerlin et al., 2012; Moise et al., 2014). A pesar de la clara compartimentalización o independencia de cada ruta, se ha demostrado que existe comunicación y complementariedad parcial entre ambas (Nagata et al., 2002; Hemmerlin et al., 2003; Rodríguez-Concepción, 2010; Heinig et al., 2013; Gutensohn et al., 2013).

La ruta MEV genera precursores principalmente para la síntesis de esteroles, brasinosteroides y sesquiterpenos, entre otros. La ruta MEP, por su parte, es la principal fuente de precursores para la síntesis de carotenoides y hormonas derivadas (estrigolactonas y ácido abscísico), así como también de monoterpenos, diterpenos (giberelinas, fitoalexinas), citoquininas, clorofilas, tocoferoles, plastoquinonas, etc. (Bouvier et al., 2005; Rodríguez-Concepción, 2010). La ruta MEV se inicia con la condensación de gliceraldehído-3-fosfato con una hidroxietil tiamina (derivada de piruvato) para formar dioxixilulosa-5-fosfato (DXP), mediante la acción de la enzima dioxixilulosa-5-fosfato sintasa (DXS), punto clave regulador del flujo en la ruta (Estévez et al., 2001; Rodríguez-Concepción et al., 2001; Peng et al., 2013; Wright et al., 2014). A continuación, el DXP es reducido por la enzima DXP reducto isomerasa (DXR) para formar metileritritol fosfato (MEP) y tras cuatro reacciones sucesivas se obtiene el hidroximetilbutenil difosfato (HMBPP) el cual, mediante la acción de la enzima HMBPP reductasa (HDR), también descrita como un paso regulador de flujo en la ruta (Botella-Pavía et al., 2004), forma los isómeros, IPP y DMAPP (Rodríguez-Concepción, 2010; Fig. 7). A continuación, la adición de tres moléculas de IPP a una de DMAPP origina geranil geranil difosfato (GGPP), reacción catalizada por la enzima GGPP sintasa (GGPPS), compuesta por una familia con numerosas isoformas (Beck et al., 2013; Coman et al., 2014). El GGPP es el precursor directo no sólo de los carotenoides, sino también de la biosíntesis de giberelinas, plastoquinonas, clorofilas y tocoferoles, entre otros (Bouvier et al., 2005; Rodríguez-Concepción, 2010).

1.3.3.2. Síntesis de carotenos y xantofilas

La ruta de biosíntesis de carotenoides en plantas se inicia con la unión de dos moléculas de GGPP para formar el carotenoide incoloro fitoeno, mediante la acción de la enzima fitoeno sintasa (PSY), paso clave y regulador de la ruta (Fraser et al., 1995; Fray et al., 1995; Maass et al., 2009; Zhang et al., 2009). En la mayoría de especies vegetales existen diferentes isoformas de *PSY*, cuya expresión génica varía en función del tejido y del momento de desarrollo (Fanciullino et al., 2007; Zhang et al., 2009; Fantini et al., 2013; Walter et al., 2014). El fitoeno es convertido en fitoflueno, también incoloro, mediante la acción de la fitoeno desaturasa (PDS), que es a su vez transformado por esta misma enzima en ζ-caroteno, de coloración amarillo pálido. Esta molécula sufre a continuación una isomerización y una instauración, por las enzimas ζ-caroteno isomerasa (ZISO) y ζ-caroteno desaturasa (ZDS), respectivamente. Las desaturasas PDS y ZDS introducen enlaces carbono-carbono en configuración *cis*-, siendo necesaria la intervención de una isomerasa, la CRTISO, para formar el all-*trans*-licopeno, el último caroteno lineal de la ruta, de coloración roja característica (Isaacson et al., 2004; DellaPenna y Pogson, 2006; Moise et al., 2014; Giuliano, 2014; Fig. 7).

A partir del licopeno, la ruta se ramifica en dos, la rama β,ε-, que es especialmente activa en tejido cloroplástico, y la rama β,β, que predomina en tejidos coloreados (Fig. 7). La molécula de licopeno sufre la ciclación de sus dos extremos de forma secuencial para dar lugar a anillos β-ionona por acción de la enzima β-licopeno ciclasa (βLCY), formando el β-caroteno, primer carotenoide de color naranja. La enzima βLCY esta codificada por un único gen en algunas especies como *Arabidopsis*, maíz y arroz (Cunningham et al., 1996; Bai et al., 2009; Chaudhary et al., 2010), mientras que en otras plantas donde existe una síntesis muy activa de carotenoides en órganos o tejidos específicos (como en tomate, papaya, sandía, cítricos o zanahoria) existe un segundo gen, *CYCB* o *βLCY2* (Ronen et al., 2000; Alquézar et al., 2009; Devitt et al., 2010). La ciclación del licopeno ha constituido el foco de estudio de numerosos trabajos dada su implicación directa en la acumulación de licopeno en diferentes especies, entre ellas los cítricos (Alquézar et al., 2009; Mendes et al., 2011; Costa et al., 2012; Alquezar et al., 2013; Zhang et al., 2013). Una vez formado el β-caroteno con sus dos anillos en los extremos, se produce la incorporación de grupos oxigenados en la molécula mediante la acción de una hidoxilasa, la β-caroteno hidroxilasa (βCHX), formándose así las xantofilas, que son los carotenoides predominantes en tejidos coloreados de los frutos cítricos (Fig. 7). A diferencia de lo que ocurre en tejidos coloreados, en tejidos verdes predomina la rama β,ε-, en donde la ciclación del licopeno mediante la acción de la βLCY y la εLCY deriva en la formación de α-caroteno, que es hidroxilado por la ε- y βCHX para dar lugar a α-criptoxantina y posteriormente luteína, esta última xantofila típica de tejidos cloroplásticos (Fig. 7; DellaPenna et al., 2006).

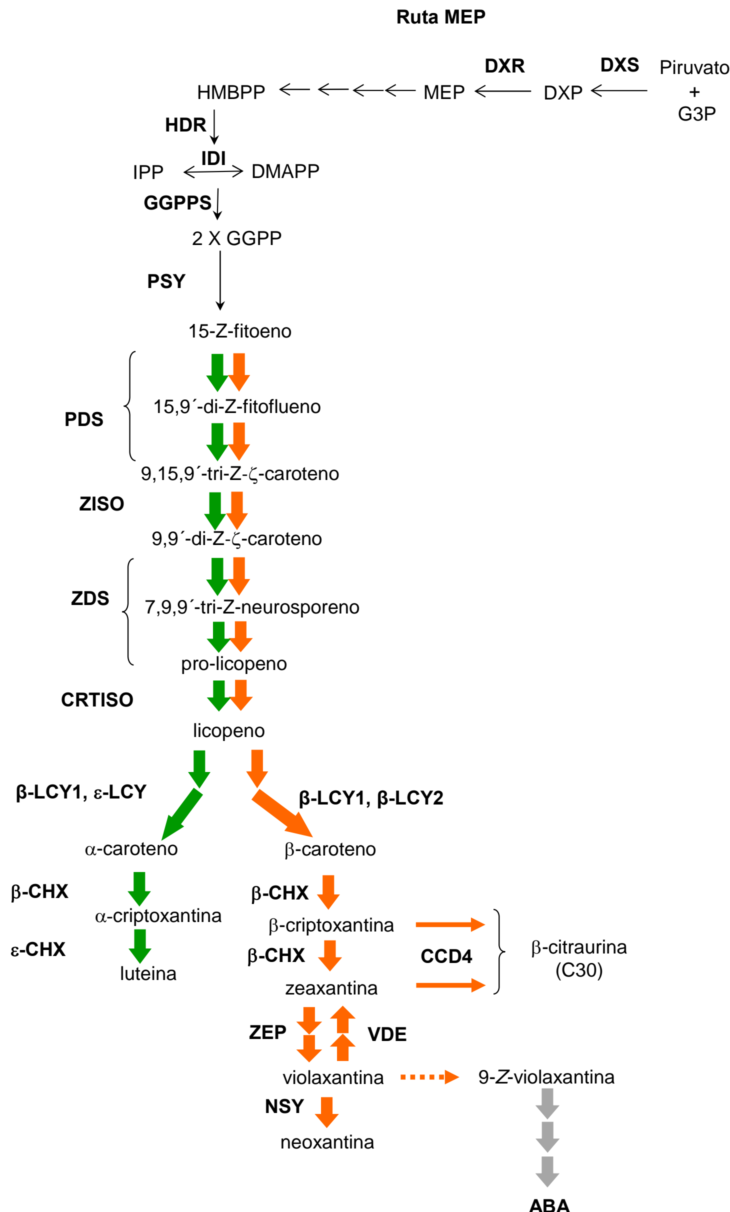

Figura 7. Esquema de la ruta de la biosíntesis de carotenoides en los frutos cítricos. Las iniciales de las enzimas son las siguientes: G3P-Gliceraldehído 3-fosfato (G3P), dioxi xilulosa-5-fosfato sintasa (DXS), dioxi xilulosa-5-fosfato (DXP), DXP reducto isomerasa (DXR), metil eritritol fosfato (MEP), hidroximetil-butenil difosfato (HMBPP), HMBPP reductasa (HDR), IPP/DMAPP isomerasa (IDI), dimetilalil difosfato (DMAPP), isopentil difosfato (IPP), geranil geranil difosfato (GGPP), GGPP sintasa (GGPPS), fitoeno sintasa (PSY), fitoeno desaturasa (PDS), ζ-caroteno isomerasa (ZISO), ζ-caroteno desaturasa (ZDS), licopeno ciclasa β (β-LCY1, β-LCY2), licopeno ciclasa ε (ε-LCY), β-caroteno hidroxilasa (β-CHX), ε-caroteno hidroxilasa (ε-CHX) zeaxantina epoxidasa (ZEP), violaxantina de-epoxidasa (VDE), neoxantina sintasa (NSY), 9-*Z*-epoxicarotenoide dioxigenasa (NCED), ácido abscísico (ABA). Las flechas naranjas indican la parte de la ruta que predomina en frutos coloreados (rama β,β-), mientras que las flechas verdes indican la rama predominante en frutos verdes (rama β,ε-). Flechas grises a continuación de la ruta de carotenoides indican varios pasos sucesivos hasta la síntesis de la fitohormona ABA. Adaptado de Rodrigo et al. (2013a).

1.3.3.3. Los plástidos: los orgánulos que sintetizan y acumulan carotenoides

En los tejidos verdes de los frutos durante las etapas del crecimiento y desarrollo previas a la maduración, los carotenoides se sintetizan y acumulan en los cloroplastos, mientras que en los frutos maduros y coloreados, se acumulan en los cromoplastos. Los carotenoides pueden sintetizarse en cualquier tipo de plástidos, a excepción de los proplastidios, incluidos los leucoplastos de las raíces, los etioplastos típicos de tejidos vegetativos que crecen en oscuridad o los amiloplastos, característicos en tejidos que acumulan almidón, como granos o tubérculos (Howitt y Pogson, 2006). En los cloroplastos, la mayoría de los carotenoides se encuentran formando parte de los fotosistemas integrando complejos proteicos anclados en las membranas (Havaux, 1998; Vishnevetsky et al., 1999). Durante el cambio de color de los frutos se produce la transformación de cloro- a cromoplastos, que son las estructuras especializadas en sintetizar y acumular grandes cantidades de carotenoides en complejos lipoproteicos especializados (Howitt et al., 2006; Cazzonelli y Pogson, 2010; Egea et al., 2010; Li et al., 2013; Nogueira et al., 2013). Los cambios más importantes durante la transición de cloro- a cromoplastos consisten en la desintegración de las membranas tilacoidales que conforman las granas y sirven de anclaje de las clorofilas, y la desaparición de los gránulos de almidón, que son abundantes en los cloroplastos (Li y Yuan, 2013).

Las estructuras que desarrollan los plástidos para acumular carotenoides varían notablemente no sólo entre las especies vegetales sino también en función del tejido u órgano de la planta. En general, los cromoplastos se clasifican en globulares, tubulares, membranosos, fibrilares o cristalinos, en función de la sub-estructura predominante (Sitte et al., 1980; Gross, 1987; Egea et al., 2010; Li et al., 2013). También se ha descrito una estrecha relación entre el carotenoide predominante y el tipo de estructura desarrollada para su acumulación (Jeffery et al., 2012; Schweiggert et al., 2012). Así, la modificación del tipo de carotenoide más abundante en los plástidos puede alterar el tipo de estructuras para su acumulación. Esto ha sido evidente en frutos tratados con inhibidores de la biosíntesis de carotenoides, como el 2-(4-clorofreniltiol) trietilamina hidrocloruro (CPTA), un inhibidor de la actividad licopeno ciclasa que produce la acumulación de licopeno (Coggins et al., 1970) y favorece el desarrollo de nuevas estructuras cristalinas que corresponden a la acumulación de cristales de licopeno (Simpson y Lee, 1977; Deruère et al., 1994; Al-Babili et al., 1999). De forma similar, la modificación biotecnológica de la biosíntesis de carotenoides en tomate mediante la sobre-expresión de determinados genes de la ruta provocó cambios en las sub-estructuras de acumulación de estos compuestos (Nogueira et al., 2013). En este sentido, se ha demostrado que el fitoeno se acumula preferentemente en plastoglóbulos, sub-estructuras globulares típicas de biosíntesis y acumulación de carotenoides (Nogueira et al., 2013), y que la acumulación de isómeros *trans*- del β-caroteno y licopeno ocurre preferentemente en estructuras cristalinas desarrolladas para aumentar la estabilidad de estos compuestos (Vasquez-Caicedo et al., 2006; Simkin et al., 2007; Schweiggert et al., 2011; Nogueira et al., 2013). El tipo de sub-estructura, además de relacionarse directamente con los tipos de pigmentos, puede afectar también la biodisponibilidad de los carotenoides (Jeffery et al., 2012; Schweiggert et al., 2012; Schweiggert et al., 2014).

Existen otros factores que pueden intervenir o influir en la acumulación de carotenoides en las diferentes sub-estructuras. Así, la mutación en el gen *orange* (*Or*) identificada en coliflor provocó la aparición de inflorescencias de color naranjas debido a una acumulación de β-caroteno, ya que promueve la diferenciación de sub-sestructuras para acumular carotenoides sin modificar el perfil transcripcional de los genes de biosíntesis de carotenoides (Li et al., 2001; Lu et al., 2006). Resultados similares se observaron al sobre-expresar el mismo gen en boniato, aunque en este caso sí se observó una mayor expresión de genes de biosíntesis de carotenoides (Kim et al., 2013). En el caso de *Arabidopsis thaliana*, la sobre-expresión de este gen provocó una maduración precoz de los plástidos permitiendo el desarrollo de estructuras cristalinas y la acumulación de cantidades crecientes de carotenoides (Bai et al., 2014). Este grupo de proteínas han sido a su vez sugeridas recientemente como reguladores post-transcripcionales del nivel de proteína y actividad de la enzima *PSY* en *Arabidopsis* (Zhou et al., 2015). Estos resultados sugieren que un aumento en la acumulación de carotenoides puede estar vinculado a dos mecanismos principales, que actuarían conjunta o individualmente según el órgano y la especie: 1) un aumento en la expresión de los genes de biosíntesis de carotenoides que generan un mayor flujo en la ruta (Fray et al., 1995; Maass et al., 2009) y 2) desarrollo de las sub-estructuras que favorecen la acumulación de estos pigmentos de los cromoplastos (Li et al., 2001; Cazzonelli y Pogson, 2010; Rodríguez-Concepción y Stange, 2013; Kim et al., 2013; Bai et al., 2014). A su vez, en algunos casos se ha demostrado que ambos procesos están estrechamente relacionados, ya que pueden ocurrir de forma conjunta (Kim et al., 2013).

Además del gen *Or*, existen otras proteínas estructurales que se han relacionado directamente con cambios en la transición de cloro- a cromoplastos. Este es el caso de las proteínas de choque térmico (heat shock proteins, HSPs) y las fibrilinas/plastoglobulinas (FIBs). Se ha descrito un aumento en la expresión de genes de HSPs, principalmente el gen *HSP21*, durante la maduración de frutos de tomate y que redundó en un aumento significativo en la acumulación de carotenoides (Neta-Sharir et al., 2005). Estas proteínas son muy abundantes en el proteoma de los cromoplastos aislados de vesículas de zumo de cítricos (Zeng et al., 2011) y su expresión diferencial se ha relacionado con cambios en la coloración entre variedades (Pan et al., 2012). De forma similar, las FIBs forman parte de los plastoglóbulos, estabilizando y dando lugar a la formación de estas sub-estructuras cromoplásticas (Deruère et al., 1994; Singh y McNellis, 2011), y que parecen estar también implicadas en la maduración y diferenciación de cromoplastos en tomate (Simkin et al., 2007). Además, el silenciamiento de FIBs mediante RNAi en pétalos de flores de tomate provocó una reducción del 30% del contenido de carotenoides (Leitner-Dagan et al., 2006). Estas proteínas también han sido asociadas con cambios en la coloración de los frutos cítricos (Moriguchi et al., 1998; Pan et al., 2012).

1.3.4. Contenido y composición de carotenoides en los frutos cítricos

Los carotenoides son los pigmentos responsables de la coloración característica de los frutos cítricos. En los frutos inmaduros, de color verde y con presencia de clorofilas, se acumulan importantes cantidades de carotenoides, cuya composición es, en general, similar a la de otros tejidos fotosintéticos, donde la luteína es la xantofila más abundante, y hay concentraciones relevantes de α- y β-caroteno, y de las xantofilas zeaxantina, neoxantina y violaxantina

(principalmente en la configuración *trans*) y en algunos casos se ha descrito la presencia de α-criptoxantina. Durante el cambio de color de los frutos se activa la rama β,β- de la ruta de biosíntesis de carotenoides, de forma paralela a una reducción en carotenoides cloroplásticos de la rama β,ε- (Fig. 7). En los tejidos coloreados de los frutos cítricos existe una gran diversidad en el contenido y composición de carotenoides, lo que determina la variedad de coloraciones tanto en el flavedo como en la pulpa, desde el amarillo pálido de algunos pomelos o más intenso en los limones, a las diferentes tonalidades naranja de naranjas y mandarinas, hasta el rojo en algunas naranjas y pomelos (Kato et al., 2004; Fanciullino et al., 2006; Xu et al., 2006; Fanciullino et al., 2008; Kato, 2012; Rodrigo et al., 2013a).

El aumento en la concentración de carotenoides durante la maduración de los cítricos ocurre coordinadamente con un incremento en la expresión de los genes de su biosíntesis, principalmente los de la rama β,β- (Fig. 7) (Kato et al., 2004; Rodrigo et al., 2004; Kato, 2012; Rodrigo et al., 2013a). En general, las β,β-xantofilas, principalmente violaxantina, β-criptoxantina y sus ésteres, son los carotenoides más comunes en la mayoría de los cítricos coloreados (Gross, 1987). Cabe destacar especialmente la presencia de apocarotenoides C_{30} específicos del género en la piel de los frutos cítricos, siendo la β-citraurina el más importante y responsable de la intensa coloración naranja-rojiza en mandarinas, naranjas y algunos híbridos (Ma et al., 2013; Rodrigo et al., 2013b). En general, el contenido de carotenoides en los frutos cítricos es entre 3 y 15 veces mayor en la piel que en la pulpa (Alquézar et al., 2008b). Las naranjas acumulan entre 40 y 120 µg/g PF carotenoides totales en la piel y entre 4 y 28 µg/g PF en la pulpa. El mayor porcentaje del contenido carotenoides se debe a β,β-xantofilas (80-90% del total), siendo la 9-*Z*-violaxantina y la β-criptoxantina las principales en frutos coloreados (Kato et al., 2004; Rodrigo et al., 2004; Alquézar et al., 2008b; Kato, 2012; Rodrigo et al., 2013a). Por otra parte, en las mandarinas el contenido total de carotenoides en la piel oscila entre 25 a más de 300 µg/g PF y en la pulpa entre 18 y 34 µg/g PF en mandarina común, clementinas y satsumas, mientras que asciende a valores entre 39 y 45 µg/g PF en algunos híbridos. Tanto el contenido como la composición de carotenoides varía notablemente entre las diferentes especies y variedades de mandarinas (Alquézar et al., 2008b). En general, la composición en este grupo es similar a la de naranjas pero más compleja, ya que se han identificado más de 20 carotenoides diferentes, predominando las β,β-xantofilas (68-90%), principalmente 9-*Z*-violaxantina y cantidades importantes de β-criptoxantina y del apocarotenoide β-citraurina (Matsumoto et al., 2007; Matsumoto et al., 2009; Kato, 2012; Rodrigo et al., 2013a; 2013b). Por otro lado, los limones acumulan importantes cantidades de carotenoides incoloros en la piel (desde 1,4 a 90,0 µg/g PF, principalmente de fitoeno y fitoflueno) y muy pocos carotenoides en la pulpa (0,3-6,5 µg/g PF), de acuerdo con su coloración verdosa a amarilla de diferente intensidad (Alquezar et al., 2008b; Kato, 2012; Rodrigo et al., 2013a). De forma similar, los pomelos blancos tienen un contenido muy bajo en carotenoides, tanto en la piel como en la pulpa (menos de 4.0 µg/g PF), principalmente de fitoeno, fitoflueno, y también cantidades menores de 9-*Z*-violaxantina (Alquezar et al., 2008b; 2013). En pumelos blancos la composición es similar y los contenidos varían entre 5 y 19 µg/g PF en la piel y 0.4-1.0 en la pulpa (Alquézar et al., 2008b).

La existencia de mutaciones naturales o inducidas ha favorecido la variabilidad en el color de los frutos en las diferentes especies cítricas. Un ejemplo característico lo constituyen las

naranjas, donde se han identificado mutantes de pulpa de color rojo, como la naranja Cara Cara, Hong Anliu y Sarah, originadas a partir de los parentales Washington Navel, Anliu y Shamouti, respectivamente, que presentan la capacidad de acumular grandes cantidades de carotenos lineales, principalmente fitoeno y licopeno en la pulpa (Monselise y Halevy, 1961; Xu et al., 2006; Liu et al., 2007; Alquezar et al., 2008a). Se han descrito nuevas mutaciones de naranjas de pulpa roja, procedentes de programas de mejora de Sudáfrica (Cuellar et al., 2014) o de Brasil (Rodrigo, comunicación personal), que están siendo objeto de estudio actualmente. Además de las naranjas, también se han descrito variedades de pumelo de tonalidad roja en la pulpa, como es el caso de las variedades Chandler, Chuzhou Early Red, Fengdu y Guanxi, debido a la acumulación de licopeno (Xu et al., 2006; Alquézar et al., 2008b).

Dicha variabilidad natural también ha ocurrido entre los pomelos, con la diferencia de que en esta especie existen variedades con la capacidad de acumular grandes cantidades de fitoeno y licopeno no solo en la pulpa, sino también en la piel (Xu et al., 2006; Costa et al., 2012; Alquezar et al., 2013). Tal es el caso de los pomelos rojos Star Ruby (Alquezar et al., 2013), Flame (Costa et al., 2012) o Ruby Red (Red Blush) o Thompson (Marsh Pink) (Xu et al., 2006). Estas variedades acumulan importantes cantidades de carotenoides en la piel que oscilan entre 16 y 62 μg/g PF y entre 1 y 53 μg/g PF en la pulpa, predominando en ambos tejidos la presencia de los carotenos lineales fitoeno, fitoflueno y licopeno (Xu et al., 2006; Alquezar et al., 2008b; 2013; Rodrigo et al., 2013a).

El licopeno es un carotenoide lineal que destaca por su elevada capacidad antioxidante, dada la presencia de 11 dobles enlaces conjugados en la molécula (Di Mascio et al., 1989; Singh y Goyal, 2008). Esta característica hace que tenga el doble de capacidad que el β-caroteno para secuestrar $^1O_2$ y 10 veces respecto al α-tocoferol (Di Mascio et al., 1989; Aizawa et al., 2011). Los mecanismos bioquímicos por los que se produce esta acumulación en los frutos cítricos están siendo objeto de estudio en los últimos años (Alquezar et al., 2008a; Alquézar et al., 2009; Xu et al., 2009; Pan et al., 2009; Mendes et al., 2011; Costa et al., 2012; Pan et al., 2012; Alquezar et al., 2013) aunque aún se desconocen las causas que llevan a su acumulación en esta especie**.** Los diversos trabajos desarrollados hasta el momento sugieren diferentes mecanismos implicados proceso. Por un lado, en pomelos rojos o rosados se ha descrito que una disminución de la expresión y actividad del gen de la β-licopeno ciclasa 2 (*βLCY2*) durante la maduración del fruto, respecto a las naranjas o los pomelos blancos, podría ser responsable de la acumulación de licopeno en esta especie (Alquézar et al., 2009; Mendes et al., 2011; Costa et al., 2012; Alquezar et al., 2013). Por otro lado en las naranjas, el análisis de la expresión de genes de biosíntesis de carotenoides en la variedad roja Cara Cara revela que las diferencias respecto a la línea parental parecen situarse en una mayor expresión de genes de la ruta MEP (Alquézar et al. 2008a; Fanciullino et al., 2008). En el estudio de los cambios en el proteoma y transcriptoma en la pulpa del mutante de naranja Hong Anliu, que también acumula licopeno, se ha descrito una menor expresión del gen *βLCY2* y también una mayor actividad/expresión de enzimas/genes relacionados con sistemas antioxidantes (Pan et al., 2009; Yu et al., 2012), sugiriendo que la acumulación de licopeno podría potenciar la capacidad antioxidante del tejido.

1.3.5. Regulación de la síntesis de carotenoides

1.3.5.1. Factores endógenos: maduración de los frutos

La regulación de la biosíntesis de carotenoides en los frutos es altamente compleja, donde se coordina el programa genético del desarrollo que conduce al cambio de color durante la maduración, con la influencia de diferentes factores ambientales. El fruto de tomate ha sido el sistema modelo donde más extensamente se han estudiado los cambios transcripcionales durante la maduración, debido principalmente a las posibilidades de manejo y el relativamente corto ciclo de vida, a las características genéticas y a la disponibilidad de mutantes y variantes con características alteradas. En este fruto se han identificado y caracterizado la mayoría de genes estructurales de la biosíntesis de carotenoides, así otros factores accesorios y complementarios a la ruta, y se han analizado sus patrones de expresión y cómo se coordinan con los cambios en la coloración y en la acumulación de estos compuestos. En tomate, el inicio del cambio de color del fruto durante la maduración parece ser paralelo a una inducción importante de la enzima *PSY1* y una represión en la expresión de los genes *βLCY* y *εLCY*, que favorece la acumulación de fitoeno y licopeno (Giuliano et al., 1993; Fraser et al., 1994). Se ha descrito también la inducción de la ruta MEP durante la maduración, principalmente a nivel de las enzimas *DXS* y *HDR*, cuya mayor expresión está directamente relacionada con el aumento en el contenido de carotenoides, mientras que la enzima *DXR* parece presenta un papel constitutivo y no limitante (Rodríguez-Concepción et al., 2001; Botella-Pavía et al., 2004; Enfissi et al., 2005). Adicionalmente, distintos trabajos también han sugerido la existencia de otros mecanismos de regulación post-transcripcional y post-traduccional durante la acumulación de carotenoides en los frutos de tomate (Schofield y Paliyath, 2005; Enfissi et al., 2010; Kilambi et al., 2013; Gupta et al., 2014).

En el caso de los frutos cítricos, la expresión de los genes de biosíntesis de carotenoides es también menor en frutos verdes y se incrementa significativamente durante el cambio de color, principalmente el gen *PSY*, que tiene un papel clave regulando la entrada a la ruta, así como también los genes *PDS, ZDS*, *LCY*s y *βCHX,* que conducen finalmente a la formación de las β,β-xantofilas conjuntamente con una reducción en la expresión del gen *εLCY*. Esta tendencia en los perfiles transcripcionales se observa tanto en la piel como en la pulpa de todas las especies cítricas, aunque los cambios son mucho menos pronunciados en la pulpa (Kato et al., 2004; Rodrigo et al., 2004; Alquézar et al., 2008a, 2008b; Distefano et al., 2009; Kato, 2012; Alquézar et al., 2013; Rodrigo et al., 2013a; Wei et al., 2014).

Existen a su vez cambios relativos a la expresión de estos genes entre frutos de distintas variedades durante la maduración, lo que puede contribuir a las notables diferencias en la coloración y perfil de carotenoides entre las mismas (Alquézar et al., 2008a, 2008b; Kato, 2012). En el caso de la enzima encargada de ciclar el licopeno, se ha descrito la presencia de dos isoformas diferentes en los frutos cítricos; la *βLCY1*, cuya expresión es mayor en tejidos inmaduros y la *βLCY2*, específica de tejidos coloreados, cuya expresión aumenta de forma significativa durante la maduración. A su vez, el gen *βLCY2* presenta dos alelos (*a* y *b*), ambos con actividad licopeno ciclasa, pero ensayos funcionales demuestran que el alelo *βLCY2b* presenta una actividad ciclasa casi nula, siendo la variante más abundante en los frutos de pomelo (Alquézar et al., 2009;

Alquezar et al., 2013). A su vez, el aumento en la expresión de este gen durante la maduración es de menor magnitud en pomelos que en naranjas (Alquézar et al., 2009). Además de la ruta de biosíntesis de carotenoides, también se ha descrito una inducción en los genes de la ruta MEP durante la maduración de los frutos, principalmente *DXS, HDS* y *HDR* (Alquezar et al., 2008a).

1.3.5.2. Factores ambientales: efecto de la luz en la biosíntesis de carotenoides

Entre los factores ambientales que afectan la síntesis y acumulación de carotenoides en los diferentes órganos y tejidos de las plantas, incluyendo los frutos cítricos, destacan la luz y la temperatura (Ruiz-Sola y Rodríguez-Concepción, 2012; Cronje et al., 2013; Rodrigo et al., 2013a; Porras et al., 2014). Se ha descrito, en general, un efecto positivo de la luz en la acumulación de carotenoides, tanto en tejidos reproductivos como vegetativos (Pizarro y Stange, 2009). Sin embargo, existen claras excepciones como es el caso de la zanahoria (raíz) en donde la ausencia de luz es fundamental para inducir la acumulación de grandes cantidades de carotenoides, principalmente β y α-caroteno, efecto que se revierte en presencia de luz (Fuentes et al., 2012; Rodríguez-Concepción y Stange, 2013).

En general, la mayor acumulación de carotenoides en condiciones de luz está asociada a una mayor expresión de los genes de la ruta de biosíntesis, así como también de los genes de la ruta MEP (Toledo-Ortiz et al., 2010; Ruiz-Sola et al., 2012; Gupta et al., 2014). Se ha descrito que esta regulación esta mediada por factores de transcripción PIFs (del inglés phytochrome interacting factor) que regulan negativamente la expresión del gen *PSY* y son reprimidos por la presencia de luz (Toledo-Ortiz et al., 2010). Así pues, los PIFs participan directamente de la regulación de la biosíntesis de carotenoides a través del control de la expresión de la *PSY* y también la *DXS* (Toledo-Ortiz et al., 2010; Leivar y Quail, 2011; Toledo-Ortiz et al., 2014). Este mecanismo regulador actúa en combinación con el factor de transcripción *HY5* tipo b-ZIP, un potente antagonista de los PIFs, que forma parte de un módulo dinámico de activación-represión en respuesta a la luz y a la temperatura (Toledo-Ortiz et al., 2014). De forma similar, se ha demostrado que la expresión de la enzima *βLCY* está regulada de forma positiva por la luz en tejidos vegetativos (Fu et al., 2013). También se ha descrito la posible existencia de mecanismos de regulación post-transcripcional en respuesta a la luz tanto en la ruta de carotenoides como en la MEP (Schofield et al., 2005; Hemmerlin et al., 2012; Hemmerlin, 2013).

En el caso de los cítricos, se ha observado que los frutos que crecen en el exterior del árbol desarrollan una coloración más intensa y acumulan mayores cantidades de carotenoides totales que aquellos localizados en el interior del árbol (Cronje et al., 2011; 2013). De forma similar, frutos provenientes de huertos con una menor densidad de plantación y, por tanto, una penetración de la luz más eficiente, desarrollaron una coloración más intensa (Boswell et al., 1982). Otros experimentos han mostrado que al suprimir la exposición a la luz durante un periodo de tiempo prolongado mediante el embolsado, los frutos mostraron una reducción significativa en la coloración de la piel (Uchida et al., 1985) y, de forma similar, los frutos del interior de la copa del árbol también presentaron una reducción en el color directamente proporcional a la radiación

recibida (Jifon y Syvertsen, 2001). En naranjas del grupo Navel expuestas únicamente al 5% de la radiación normal, se produjo una reducción del 42% en el contenido de carotenoides en la piel en comparación con frutos crecidos en condiciones lumínicas normales (Lewis y Coggins, 1964). Sin embargo, los mecanismos bioquímicos y moleculares que regulan este fenómeno en las diferentes variedades cítricas son todavía desconocidos.

2. OBJETIVOS

Con los antecedentes anteriores, en este trabajo de Tesis Doctoral se plantea el siguiente objetivo general:

Estudiar la implicación de los carotenoides en la calidad postcosecha de los frutos de pomelo, especialmente durante la conservación a bajas temperaturas, y analizar el papel del etileno y del sistema antioxidante en este proceso. Este objetivo se complementa con el estudio de los cambios fisiológicos, moleculares y estructurales que ocurren en frutos cítricos en diferentes condiciones ambientales y en frutos con diferente pigmentación.

Para abordar este objetivo se desarrollarán los siguientes objetivos parciales:

1 Caracterizar el papel de los carotenoides, y en particular del licopeno, en la susceptibilidad a los daños por frío durante de la conservación refrigerada de frutos de pomelos Marsh y Star Ruby, y evaluar la implicación del etileno en el desarrollo de esta fisiopatía.

2 Explorar los cambios en el sistema antioxidante (enzimático y no enzimático) durante el almacenamiento refrigerado de frutos de pomelo con diferente susceptibilidad a desarrollar daño por frío.

3 Caracterizar las bases fisiológicas y moleculares que estimulan la acumulación del caroteno licopeno en frutos de pomelo rojo en condiciones ambientales de oscuridad.

4 Estudiar la relación entre la composición de carotenoides y los cambios estructurales en los plástidos durante la maduración de frutos cítricos con diferente pigmentación.

3. RESULTADOS

3.1. CAPÍTULO I.

Involvement of lycopene in the induction of tolerance to chilling injury in grapefruit

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Abstract

Grapefruit are among the *Citrus* more sensitive varieties likely to develop chilling injury (CI) symptoms during postharvest storage at low temperatures. Comparative observations of the incidence of CI in fruit of the white Marsh (MSH) and the red Star Ruby (SR) grapefruit during postharvest storage at 2 ºC plus 7 days at 20 ºC to simulate shelf-life revealed that 1) the former was consistently more sensitive to CI, developing cold damage uniformly throughout the whole rind surface, and 2) more strikingly, CI symptoms in fruit of SR grapefruit were restricted to the yellow areas of the rind and the red-colored zones were almost absent of cold damage. This tolerance to CI in red flavedo was associated with high carotenoid (x2) and lycopene (x14) contents, as compared with yellow-colored flavedo. Absence of chilling damage in red areas of SR grapefruit rind was confirmed by cellular ultrastructure observations, in which these epidermal cells were intact, with a well-defined structure and compact vacuoles filled with content. Cells of yellow-colored tissue developing CI, were collapsed, with a contracted vacuole and shrinking organelles. To explore whether the tolerance to CI in red areas of grapefruit rind was due to an elevated lycopene concentration, chemical and environmental stimulation of this carotenoid was performed in fruit of both grapefruit varieties. Application of the inhibitor of the lycopene cyclase activity, CPTA (2-(4-chlorophenylthio) triethylamine hydrochloride) induced red coloration, increased lycopene accumulation (x32) and significantly delayed development of CI symptoms in the rind the CI-sensitive MSH. Bagging of SR grapefruit enhanced a homogenous red coloration and substantially induced lycopene accumulation (x75). CI symptoms in bagged fruit were notably delayed and reduced, as compared with non-bagged yellow fruit, upon subsequent storage at 2 ºC for up to 58 days and 7 days at 20 ºC. Analysis of the expression of ethylene biosynthetic genes (*ACS1*, *ACS2* and *ACO*) revealed a significant induction in chilling-damaged tissue of both varieties that was almost absent in red chilling-tolerant tissue. Similarly, accumulation of transcripts of the ethylene receptors *ETR1* and *ETR3* were also associated with chilling damage, but a cold factor appears to also mediate the expression of these genes. Taken together, our results indicate that high lycopene concentration appears to be responsible for the induction of tolerance to chilling in the red-colored areas of the flavedo of grapefruit during postharvest storage at low temperatures.

INTRODUCTION

Grapefruit (*Citrus paradisi* Macf.) are among the most susceptible *Citrus* species to develop CI when stored at temperatures below 8-10 ºC (Schirra, 1992; Schirra et al., 1998). CI symptoms in grapefruit are initially manifested as small brown pits on the fruit surface that progressively increase in size forming shrunken brown spots and depressed areas of different shape, that may affect large surface of the rind after prolonged cold storage periods (Schirra, 1992; Schirra et al., 1998; Lafuente and Zacarías, 2006;). Susceptibility to develop CI may depend on the grapefruit variety, growing conditions or the harvest season, as it has been demonstrated that grapefruit harvested early and later in the season are more susceptible to CI than mid-season fruit (Schirra et al., 1998; Dou, 2005).

In order to induce cold tolerance in CI-sensitive citrus fruit, various postharvest treatments have been investigated over the years. Temperature conditioning treatments before storage are among the most effective systems to induce cold tolerance and maintain citrus fruit quality during low temperature exposure (Hatton and Cubbedge, 1981; Porat et al., 2000; Biolatto et al., 2005). Conditioning treatments in grapefruit are carried out at either 21 ºC for 3 days of at 16 ºC for 7 days, although the tolerance induced to CI appears to be better by the latter treatment (Hatton and Cubbedge, 1981). Hot water dip treatments (Rodov et al., 1995; Ghasemnezhad et al., 2008), intermitent warming (Davis et al., 1973) and rinsing and brushing at temperatures of ± 60 ºC for a few seconds (Porat et al., 2000) have been also reported to be effective in inducing chilling tolerance. Moreover, other non-thermal treatments, such as waxing or modified atmosphere packaging (Porat et al., 2000), thiabendazole or imazalil dip application (Schirra et al., 2000) or various combined methods (Rodov et al., 2000) may induce, to different extents, cold stress tolerance.

Biochemical and molecular mechanisms involved in chilling-tolerance in *Citrus* fruit have been extensively studied in the last decade. Genes differentially expressed by heat-induced chilling tolerance were initially identified in the chill-sensitive 'Fortune' mandarin (Sanchez-Ballesta et al., 2003). More recently, a large number of genes induced by mild temperature conditioning (16 ºC for 7 days) or by rinsing and brushing at high temperatures (62 ºC for 20 s) followed by cold storage have been identified in grapefruit (Sapitnitskaya et al., 2006; Maul et al., 2008). Results indicated that a complex interplay of different metabolic pathways operate in the induction of cold tolerance (lipid metabolism, metallothioneins, oxidative stress, dehydrins, osmoprotectants, defense responses, among others). Each heat treatment appears to selectively induce the expression of a specific set of genes, and also to modify the expression of chilling-induced transcripts (Maul et al., 2011).

An increased ethylene production during exposure of citrus fruit to chilling temperatures has been observed in many varieties (McCollum and McDonald, 1991; Lafuente et al., 2001; Ghasemnezhad et al., 2008). The increment of ethylene is associated with an induction of *ACO* and *ACS* genes expression during cold storage (Zacarías et al., 2003; Maul et al., 2008) while temperature conditioning treatments, which reduced CI symptoms, down-regulated expression of *ACO* gene (Maul et al., 2011). These results suggest that ethylene is involved in the response of citrus fruit to postharvest cold stress, but whether ethylene is a protective defense response or a cause of CI-induced damage is not fully understood (Lafuente and Zacarías, 2006).

Fruit coloration is one of the most important attributes determining fruit quality and directly affects consumer acceptance. In *Citrus*, fruit color is determined by the specific and particular carotenoid content and composition in the rind and pulp of the different varieties (Kato, 2012; Rodrigo et al., 2013a). Carotenoids are C_{40} isoprenoid molecules formed by the condensation of two geranylgeranyl diphosphate (GGPP) molecules and play a wide range of functions in plants, transferring energy to chlorophyll, as photoprotectors, dissipating excess light energy, displaying an important role in membrane stabilization (especially in chloro- and chromoplasts), and as powerful scavengers of reactive oxygen species, protecting the cell from oxidative stress (Britton, 2008). Moreover, carotenoids are also relevant for human nutrition and health, as specific carotenoids are the precursors of vitamin A (α- and β-carotene, and β-cryptoxanthin) and display potent antioxidant properties (lycopene), having then protective effects against degenerative diseases (Rao and Rao, 2007).

Among carotenoids, lycopene is an acyclic red carotene with 13 double bounds, 11 of which are conjugated and responsible for the high antioxidant capacity of the molecule (Di Mascio et al., 1989; Krinsky, 1989; Aizawa et al., 2011;Rodrigues et al., 2012). Increments in carotenoid content in fruit and vegetables have been associated with a higher tolerance to different stresses. For example, transgenic cells of sweet potato accumulating higher amount of β-carotene were able to grow under stressful salt conditions, mainly due to an enhanced antioxidant capacity (Kim et al., 2012; Kim;Kim;et al., 2013). An increased accumulation of lycopene in tomato has also been associated with a lower incidence of chilling damage (Whitaker, 1994) and damaged fruit showed four times lower lycopene than healthy fruit (Rugkong et al., 2011). Similarly, accumulation of other potent antioxidants (anthocyanins) in transgenic tomato led to a reduction in postharvest deterioration and disease incidence during cold storage (Zhang et al., 2013).

Since the development of CI has been associated with oxidative stress in citrus fruit (Sala, 1998; Sala et al., 1999) and carotenoids display important antioxidant properties, it is reasonable to conceive that these pigments may have an active role in the protection of fruit from cold stress. Accumulation of lycopene in *Citrus* fruit is an unusual feature, restricted to few species such as grapefruit, pummelo and sweet orange mutants (Liu et al., 2007; Alquezar et al., 2008; Pan et al., 2009; Alquezar et al., 2013). Then, the objective of the present study was to test the hypothesis that accumulation of lycopene in the rind of grapefruit may protect this tissue from CI development during cold storage. To that end, the incidence of CI was recorded in fruit of two grapefruit varieties with contrasting fruit coloration and carotenoid complement: the white grapefruit Marsh (MSH), that has been demonstrated to be almost devoid of carotenoids in rind and pulp, and the red grapefruit Star Ruby (SR) that is widely recognized by its intense red coloration in the pulp and is also able to develop red color in the rind due to the accumulation of lycopene (Alquezar et al., 2013). Moreover, to corroborate the potential protection to CI induced by this carotene, total carotenoids and especially lycopene content were increased in the rind of grapefruit by chemical (CPTA application) and environmental (fruit shading) manipulation.

MATERIALS AND METHODS

Plant material, treatments and storage conditions

Fruit of the white Marsh and the red Star Ruby grapefruit (*Citrus paradisi* Macf.) were harvested from adult trees from an orchard located in Moncada, Valencia, Spain. Mature fruit were harvested at the end of January, delivered to the laboratory, inspected to be free of damage and defects, selected by uniformity of size and external color and then classified in three replicate lots of 20 fruit each. Fruit from the different experiments were stored at 2 ºC and 80-85% RH for up to 58 days with a subsequent 1 week storage at 20 ºC to simulate shelf-life conditions. At harvest and after 28 and 58 days of storage, the flavedo (colored part of the rind) was excised and immediately frozen in liquid nitrogen, ground to a fine powder and stored at -80 ºC until analysis. At each sampling date, rind color was measured using a Minolta CR-330 colorimeter on three locations around the equatorial plane of the fruit, using three replicates of 10 fruit each. Color was expressed as the *a/b* Hunter ratio. The *a/b* ratio is negative for green fruit, the zero value corresponds to yellow fruit at color break and is positive for orange to red colored fruit. Comparative evaluation of CI incidence in MSH and SR grapefruit harvested from the same orchard was done in two successive seasons.

Two different treatments were performed to induce red coloration in the rind of grapefruit. The first experimental approach was carried out using the inhibitor of lycopene cyclase activity, 2-(4-chlorophenylthio) triethylamine hydrochloride (CPTA), that promotes the accumulation of the red lycopene in plant tissues including citrus fruit (Coggins et al., 1970; Yokoyama et al., 1972). Fruit of the white MSH grapefruit were harvested in December, delivered to the laboratory and dipped in 5000 mg/L CPTA (Sagechem, China) + 0.1% Triton X-100 solution for 30 s and dried at room temperature. Control fruit were dipped in with the same conditions without CPTA. Control and CPTA-treated fruit were maintained 4 days at 20 ºC to stimulate pigment biosynthesis and accumulation, and then stored at 2 ºC for up to 58 days and 7 days of shelf-life at 20 ºC. In the second approach, SR fruit were covered at the end of July (55 mm diameter) with black plastic bags, to avoid direct sunlight exposure. Uncovered control fruit were located outside of the tree canopy, adjacent to covered fruit and exposed to normal light conditions. The effect of fruit shading on lycopene content and carotenoid accumulation in red grapefruit has been recently described (Lado et al., unpublished results). Covered (C) and non-covered (NC) fruit were harvested in December, external fruit color was determined and subsequently, stored at 2 ºC for 58 days and 7 days of shelf-life at 20 ºC. CI incidence, carotenoid composition and ethylene emission were evaluated during storage. The shading experiments were carried out in two orchards, a commercial one located in Lliria (Valencia, Spain) and a second in the *Citrus* Germplasm Bank (Moncada, Valencia, Spain), using at least 60 bagged and non-bagged fruit in each location.

Chilling injury evaluation

Fruit were inspected for CI incidence (intensity and extension of the damage) and scored on a scale from 0 (no injury) to 3 (severe injury) according to necrotic surface area and browning intensity. Results were expressed as CI index, which was calculated by adding the product of the number of fruit in each category multiplied by the score for each category and afterwards dividing

this amount by the total number of fruit evaluated (Schirra et al., 1998). Results correspond to the mean ± S.E. of four replicates of 10 fruit each.

Carotenoid extraction, identification and quantification

Flavedo pigments were extracted essentially as described previously (Alquezar et al., 2008). Samples were saponified using a 10% methanolic:KOH solution and carotenoid extracts were purified and concentrated to dryness under N_2. Extracts were kept at -20 ºC until HPLC analysis. All procedures were carried out on ice under dim light to prevent possible photodegradation, isomerisation and structural changes of carotenoids.

Individual carotenoid composition of each sample was analyzed by HPLC with a Waters liquid chromatography system equipped with a 600E pump and a model 2998 photodiode array detector, and Empower software (Waters). A C30 carotenoid column (250 × 4.6 mm, 5 µm) coupled to a C30 guard column (20 × 4.0 mm, 5 µm) (YMC Europe GMBH) was used. Samples were prepared for HPLC by dissolving the dried carotenoid extracts in $CHCl_3$: MeOH: acetone (3:2:1, v:v:v). A ternary gradient elution with MeOH, water and methyl *tert*-butyl ether (MTBE) was used for carotenoid separation as reported in previous works (Alquézar et al. 2008). The carotenoid peaks were integrated at their individual maxima wavelength and their content was calculated using the following calibration curves: β-cryptoxanthin (Extrasynthese); lutein (Sigma) for lutein and violaxanthin isomers; lycopene (Sigma); zeaxanthin (Extrasynthese); β-carotene (Sigma); 8-β-apocarotenal (Roche) for β-citraurin. Standards for calibration curves of phytoene, phytofluene and ζ-carotene were obtained from purified flavedo extracts of 'Pinalate' sweet orange fruit, which accumulate large amounts of these compounds (Rodrigo et al., 2003). Total carotenoid content was calculated as the sum of individual carotenoids. Samples were extracted twice and each analytical determination was replicated three times. All operations were carried out on ice under dim light to prevent photodegradation, isomerisation and structural changes of carotenoids.

Determination of ethylene production

Ethylene production was measured at different time intervals during storage of MSH and SR grapefruit at 2 ºC. Three replicates of four fruit each were incubated in hermetically sealed jars for 5 hours and maintained at the same storage temperature. After that period, 1 mL of air from the headspace was withdrawal with a hypodermic syringe and the ethylene content analyzed by GC. A Perkin-Elmer Autosystem (Norwalk, CT) gas chromatograph equipped with an activated alumina column and an ionization flame detector was used. The detector was stabilized at 180 ºC, the injector and column temperature was settled at 140 ºC and nitrogen was used as gas carrier. Results were expressed as the mean of three replicates ± S.E.

Quantitative real time-PCR analysis

Total RNA was isolated from the flavedo of fruit sampled at harvest and after 28 and 58 days at 2 ºC, using RNeasy Plant Mini Kit (Qiagen) and subsequently treated with DNase (DNA free, DNase treatment & removal, Ambion). The transcripts present in 2 µg of total RNA were reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen) in a total volume of 20 µL. One µL of a 5-times diluted first-strand cDNA, containing approximately 100 ng of cDNA, was used for each amplification reaction. Quantitative real-time PCR was performed on a

LightCycler 480 instrument (Roche), using the LightCycler 480 SYBRGreen I Master kit (Roche). Reaction mix and conditions followed the manufacturer's instructions. The primers employed for the amplification of each gene related to ethylene biosynthesis and perception (*ACS*-ACC synthase, *ACO*-ACC oxidase, *ETR*- ethylene response factor) are described in Alós et al. (2014). The protocol for all genes analyzed consisted of 10 min at 95 °C for pre-incubation, then 40 cycles of 10 s 95 °C for denaturation, 10 s at 59 °C for annealing and 10 s at 72 °C for extension. Fluorescent intensity data were acquired during the extension time. For expression measurements, we used the LightCycler 480 Software release 1.5.0, version 1.5.0.39 (Roche) and calculated expression levels relative to values of a reference sample using the Relative Expression Software Tool (Pfaffl et al., 2002). Normalization was performed using the expression levels of the *ACTIN* gene which primers were described in (Alós et al., 2014). For all genes analyzed, the reference sample was the expression value obtained in the flavedo from NC fruit after 58 days of storage at 2 ºC which was set at 1. Results were the average of four independent replicates.

Transmission electron microscopy (TEM)

Samples preparation for microscopy was performed at the Central Service for Support to Experimental Research (SCSIE, Universitat de Valencia). The flavedo samples were manually cut in small squares of 5 mm long x 1 mm wide, being fixed in Karnousky solution (0.5% glutaraldehyde, 2.5% paraformaldehyde and phosphate buffer 0.1 M pH 7.4) and washed afterwards two times for 10 min in PIPES buffer 0.1 M (pH 7.2). Then, samples were post-fixed for 1 h in osmium tetroxide (1% tetroxide at 50% in PIPES buffer 0.1 M) and washed for 20 min with PIPES and briefly rinsed in water. Samples were stained in uranyl acetate (2% aqueous solution) for 20 min, proceeding afterwards to dehydrate the tissue, submerging the sample in successive 10 min immersions of ethanol 30-50-70-95% and absolute ethanol at the end. After dehydration, the samples were embedded in SPURR resin, following a gradual procedure consisting of 10 min in acetonitrile for 10 min, 50:50 acetonitrile:resin overnight and finally pure resin for 6 h. The samples were placed in capsules with SPURR resin and polymerized for 20-24 h at 60 ºC. Once polymerized, the ultra-thin (60-150 µm) sections were cut in a Leica microtome (OmU3, Reichert, Austria). Sections were stained for 5 min with lead citrate (Reynold`s), afterwards proceeding to visualization in a transmission electron microscope (Hitachi H7000, Japan).

Experimental design and statistical analysis

The experimental design was completely randomized. Results were expressed as mean ± standard errors (SE) and for continuous variables with normal distribution, data were subjected to analysis of variance and differences between means determined by Tukey test, setting significance level at $p<0.05$. In the case of gene expression analysis, data were analyzed using the software REST 2009 (http://genequantification.de/rest-2009.html) to determine statistical significance. A pair-wise fixed reallocation randomization test was applied, which makes no assumptions about the distribution of the observations.

RESULTS

Incidence of chilling injury and rind ultrastructure during cold storage of Marsh and Star Ruby grapefruit

In order to evaluate the sensitivity of MSH and SR grapefruit to CI damage during cold storage, and whether the red and yellow rind tissue areas in the same SR grapefruit (Fig. 1A-B) showed a differential susceptibility to CI, incidence of the disorder was observed during two consecutive seasons. Both varieties developed CI during cold storage at 2 ºC and initial symptoms appeared earlier in MSH (14 days) than in SR (20 days; Fig. 1C-D). The first signs of CI were small shrunken pits in the rind of both grapefruit varieties that spread out on the surface and became dark and brown, forming clusters of sunken areas, affecting external appearance and, therefore, fruit commercial quality (Fig. 1A). CI incidence was similar in MSH grapefruit in the two seasons assayed, but in SR fruit, it was lower in the first season than in the second (Fig. 1C-D). After 7 days of shelf-life at 20 ºC, CI damage increased in both varieties, but it was consistently higher in MSH (around 2.0 CI Index) than in SR (about 1.5) in both seasons.

MSH grapefruit were of uniform pale-yellow coloration throughout the whole fruit surface, whereas those of SR developed red areas in the flavedo of variable extension surrounded by yellow zones (Fig. 1A). One of the most remarkable features of the comparison in CI development between MSH and SR was that while MSH developed CI symptoms homogeneously distributed throughout the whole fruit surface, in SR fruit lesions were restricted to the yellow zones whereas the red zones remained undamaged during the entire storage period (Fig. 1B). This effect was consistently observed in fruit of the different replicate samples and during the two consecutive seasons.

To confirm the absence of chilling damage in red-colored rind areas of SR grapefruit, an ultrastructural analysis of epidermal cells was performed by TEM (Fig. 2). After 58 d of storage at 2 ºC, cells of the red zones of SR were intact, with a well-defined structure and shape, a wide cell wall, undamaged membranes, compact vacuoles filled with content and also intact plastids with electron dense bodies (Fig. 2A). Structure of these epidermal cells remained similar to that of freshly harvested fruit (not shown). By contrast, yellow epidermal cells of the same SR fruit stored at 2 ºC, showing visible CI symptoms, were completely different. Cells from these yellow zones were shrunken and collapsed, irregular in shape and the vacuole was contracted and filled with dark material, while other cellular organelles were not clearly defined (Fig. 2B). These cellular cold-induced damages were similar to those observed in the epidermal cells of MSH grapefruit, also of yellow coloration (Fig. 2C). Moreover, damaged tissue showed large empty gaps between cell walls and shrunken membranes. Cell walls from CI damaged epidermal cells were thinner and showed constrictions (asterisks) compared to healthy tissue (Fig. 2B and C).

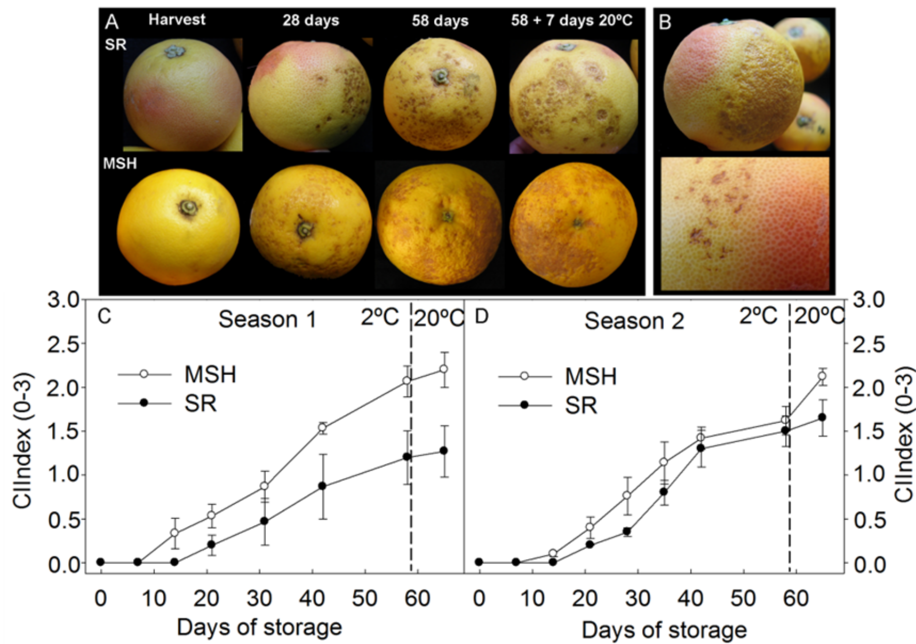

Figure 1. Morphological development of CI symptoms in the red Star Ruby (SR) and the white Marsh (MSH) grapefruit (A). Magnification of CI symptoms restricted to the yellow areas of the rind in SR grapefruit (B). Evolution of CI index in fruit of MSH and SR grapefruit stored for up to 58 days and 7 days at 20 ºC in two consecutive seasons (C and D). Dotted lines show changes on storage temperature.

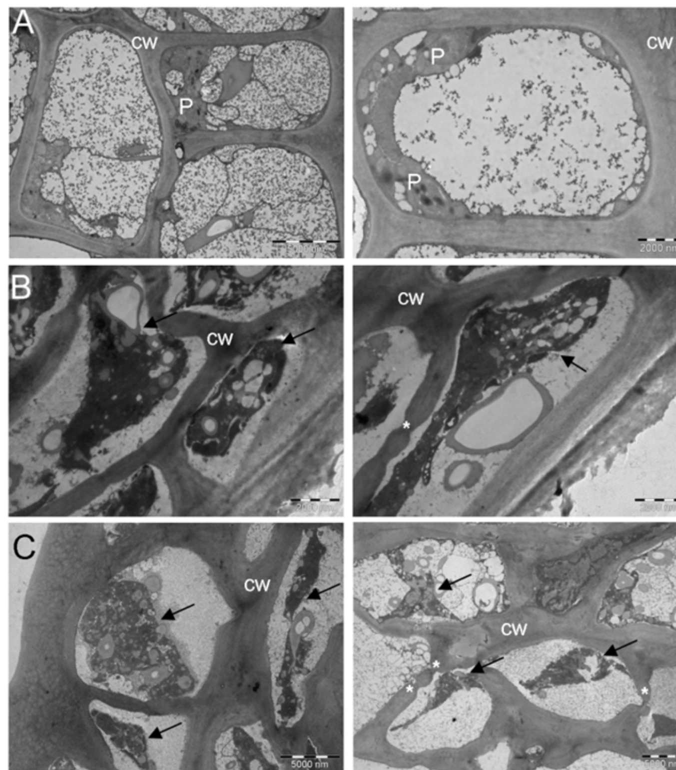

Figure 2. TEM pictures showing ultrastructure of the epidermal tissue of the red undamaged fruit SR (A), CI-damaged SR (B) and MSH (C) fruit. initial indicated: cw, cell wall; n, nucleus; P, plastids. Arrows show shrank cells with collapsed membranes where no organelles were detected and asterisks show constrictions in cell wall present in CI damaged cells.

Carotenoid content and composition in the flavedo of Marsh and Star Ruby grapefruit

Since tolerance to CI was observed in the flavedo areas of distinctive red color, as compared to the yellow CI-susceptible zones, carotenoid content and composition were determined in MSH, and in the yellow and red flavedo zones of SR. At harvest time, MSH presented an average *a/b* ratio of -0.09 while this value was of 0.16 and 0.66 in yellow and red rind of SR, respectively (Table 1). Total carotenoid content was also substantially different, accounting for 8.6 µg/g FW in MSH, 55.4 µg/g FW in yellow SR, and more than 120.0 µg/g FW in the red SR flavedo. These differences in carotenoid content were also reflected on carotenoid composition. The rind of MSH contained mainly the xanthophyll violaxanthin (6.0 µg/g FW) and the colorless phytoene (almost 2.0 µg/g FW), and minute amounts of β-carotene and phytofluene (Table 1). In both colored areas of SR grapefruit, phytoene was the most abundant carotenoid, accounting for 74% of total carotenoids in the yellow zones and nearly for 60% in the red zones. Most remarkable differences were found in lycopene and β-carotene content between both color areas which were 14- and 4-times higher, respectively, in the red than in yellow flavedo of SR (Table 1). Then, lycopene and to a lower extent β-carotene, are the carotenoids showing major differences between CI tolerant and sensitive areas in the flavedo of SR grapefruit. Based on these results, two experimental strategies were designed to explore whether these carotenoids, mainly lycopene, may be responsible for this effect.

Table 1. Rind color (a/b ratio) and carotenoids content (µg/g FW) in the rind of the yellow Marsh (MSH) grapefruit and in yellow (yellow SR) and red (red SR) areas of the rind of Star Ruby grapefruit. Data are from fruit at harvest time, and are the same fruit used for experiment of Fig. 1.

	MSH	**Yellow SR**	**Red SR**
Color (*a/b*)	-0.09± 0.02	0.16 ± 0.23	0.66 ± 0.16
Phytoene	1.91 ± 0.30	41.20 ± 3.37	72.3 ± 4.76
Phytofluene	0.11 ± 0.01	5.17 ± 0.09	15.60 ± 1.42
ζ-Carotene	-	1.21 ± 0.17	1.84 ± 0.27
Lycopene	-	1.63 ± 0.47	22.3 ± 2.26
β-carotene	0.14 ± 0.01	1.57 ± 0.09	5.60 ± 0.52
Violaxanthin	6.07 ± 0.15	4.22 ± 0.04	3.32 ± 0.21
Total carotenoids	**8.59 ± 0.19**	**55.40 ± 4.33**	**120.90 ± 0.55**

Effect of CPTA, a chemical inhibitor of lycopene cyclase activity, on chilling injury and carotenoid content and composition in Marsh grapefruit

CPTA is an inhibitor of lycopene cyclase activity that has been described to provoke lycopene accumulation and, therefore, to induce red coloration in citrus fruit (Yokoyama et al., 1972). Our rationale was that the CPTA-induced accumulation of this carotene would provide tolerance to CI in the chilling-sensitive grapefruit. Application of CPTA to MSH grapefruit caused a rapid red coloration to the rind only 4 days at 20 ºC after treatment, color changed from an *a/*b ratio lower than 0 to 0.45 (Fig. 3A and B). Control MSH non-treated fruit remained for the same period at 20 ºC and no changes in rind color or other alterations were observed. CPTA-treated and non-treated grapefruit were then stored at 2 ºC and rind color and CI index were evaluated. Rind

color remained nearly constant during storage at 2 ºC and slightly increased after simulation of shelf-life at 20 ºC (Fig. 3B). CI symptoms appeared later and developed slowly in CPTA-treated than in non-treated control fruit (Fig. 3C). At the end of the storage period and during shelf-life at 20 ºC, the CI index was significantly lower in CPTA-treated than in control fruit, reaching values of 1.1 and 1.9, respectively (Fig. 3A and C).

As expected, CPTA substantially modified carotenoid content and composition. After four days of treatment at 20 ºC, total carotenoid content increased more than 7-times in CPTA-treated fruit (from 6.3 to 48.0 µg/g FW), becoming 13-times higher than in control fruit (3.7 µg/g FW), which carotenoid levels even decreased during that period (Table 2). Lineal carotenes experienced the more relevant increment induced by CPTA (Table 2), as phytoene increased 9-times and especially lycopene which was 32-times higher than in non-treated fruit (Table 2). The content of carotenoids downstream of lycopene, like xanthophylls, remained almost constant in CPTA-treated fruit but was reduced in control grapefruit (Table 2).

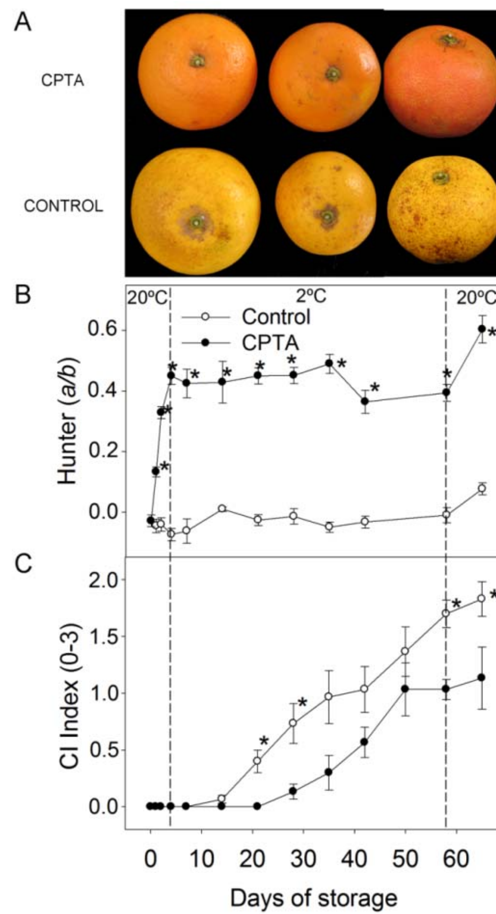

Figure 3. External appearance of Marsh grapefruit treated or non-treated with CPTA (5000 mg/L) after 28 (right), 58 (middle) and 58 days of storage at 2 ºC and 7 days at 20ºC (right) (A). Changes in rind color (*a/b* ratio) (B) and CI index (C) in Marsh grapefruit treated and non-treated CPTA (5000 mg/L) and stored at 2 ºC for 58 days and 7 days at 20 ºC. Dotted lines show changes on storage temperature. *asterisks indicate differences between CPTA treated and non-treated fruit at each sampling date ($p<0.05$, Tukey).

Table 2. Effect of CPTA application (5000 mg/L) on carotenoid content in the rind of MSH grapefruit. Fruit were treated with the CPTA, an inhibitor of lycopene cyclase activity, and incubated at 20 ºC for 4 days and after that period, rind tissue was collected for analysis of carotenoid content and composition. Carotenoid concentration (μg/g FW) is the mean ± S.E. of three determinations. Fruit were the same as used in experiment described in Fig. 3.

	Treatment		
Carotenoids	***Harvest***	***Control***	***CPTA***
Phytoene	1.14±0.19	0.97±0.03	9.18±1.41
Phytofluene	-	tr	1.03±0.28
ζ-Carotene	-	-	0.17±0.06
Lycopene	-	-	32.20±1.15
β-citraurin	0.21±0.03	tr	0.16±0.02
Zeaxanthin	0.07±0.01	-	-
Violaxanthin	4.25±0.12	2.38±0.31	4.18±0.13
Lutein	0.16±0.01	0.11±0.04	0.18±0.02
Total carotenoids	**6.35±0.13**	**3.73±0.37**	**48.00±2.96**

tr < 0.05 μg/g FW

Incidence of CI, carotenoid content and composition in shaded and non-shaded fruit of Star Ruby grapefruit

Fruit of the red SR usually develop an intense external red coloration upon shading on the tree (Lado et al., unpublished results). This effect was used to induce red coloration and to increase lycopene content in the rind in order to evaluate if these fruit presented an increased tolerance to cold-induced damage. Experiments were carried out in two locations (Lliria and *Citrus* Germplasm Bank, IVIA, Moncada, Spain), under different climatic and field conditions. Fruit were bagged in July (immature green stage) and harvested in December. At harvest time, the *a/b* ratio was 0.14 in NC fruit and near 1.0 in C ones, corresponding to yellow and red coloration, respectively (Fig. 4, Table 3).

Incidence of CI was similar in fruit from both locations but significantly lower in C than in NC fruit. CI symptoms appeared in NC fruit after 20 days of cold storage, whereas in C fruit were delayed until 40 days. Moreover, severity of the damage was higher in NC fruit, reaching a CI index higher than 2 at the end of storage in both locations. Fruit C never exceed a CI index value of 1, even after 7 days of shelf-life at 20 ºC, being commercially acceptable during all the storage period (Fig. 4 B and C).

Fruit shading caused profound changes in carotenoid content and composition and results from both locations were similar (Table 3). Total carotenoids in the rind were nearly 5-times higher in C fruit than in NC fruit. Shaded fruit presented higher carotenes content which accounted for more than 96% of total carotenoids in the rind, being phytoene (38-49%) and lycopene (38-53%) the more relevant. In accordance with the intense red color of C fruit, lycopene experienced the most remarkable increment, since in the rind of NC fruit did not exceed 0.6 μg/g FW compared to 42-43 μg/g FW in C fruit. The concentration of β-carotene was also higher in the rind of C fruit, but in contrast xanthophylls concentration was reduced (Table 3).

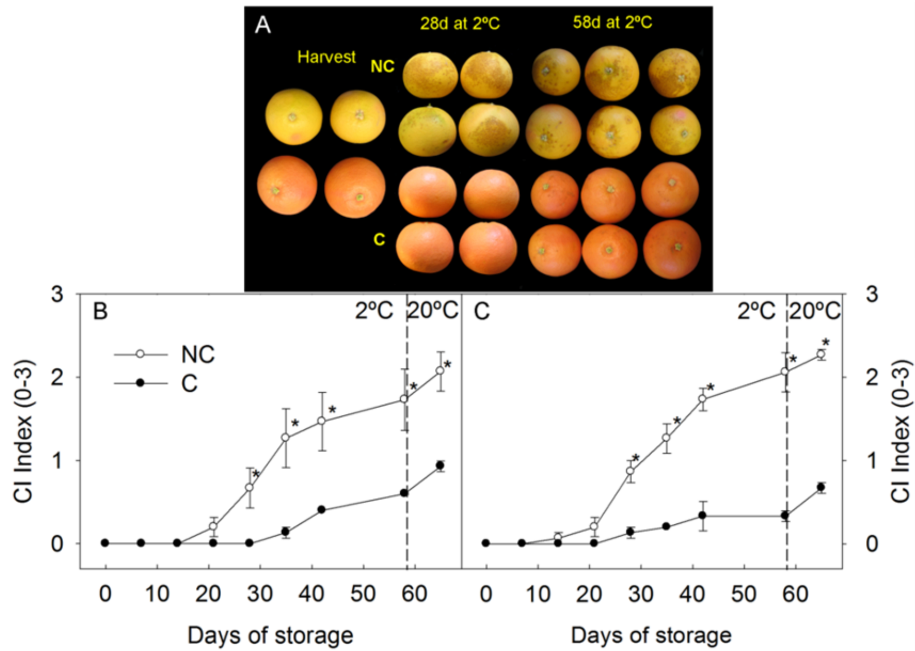

Figure 4. External appearance of Star Ruby fruit covered (C) or non-covered (NC) on the field at the harvest time and after 28 and 58 days of storage at 2 ºC (A). Changes in CI index in C and NC SR grapefruit harvested form location 1 (B, Lliria) or location 2 (C, IVIA) and stored at 2 ºC for 58 days and 7 days at 20 ºC. Dotted lines show changes on storage temperature. *asterisks indicate differences between NC and C fruit at each sampling date ($p<0.05$, Tukey).

Table 3. External color (a/b ratio) and carotenoid content in the rind (µg/g FW) in covered (C) and non-covered (NC) Star Ruby grapefruit. Fruit from two locations were covered by black plastic bags in July or remain uncovered, and harvested in December. Data are from fruit at the harvest time and the mean of three determinations ± S.E..

	Location and treatment			
	Lliria		**IVIA**	
	NC	**C**	**NC**	**C**
Color (*a/b*)	0.14±0.02	0.94±0.04	0.13±0.02	1.00±0.07
Phytoene	13.40±0.27	31.80±1.82	21.90±0.04	55.80±2.35
Phytofluene	0.97±0.02	6.59±0.45	2.18±0.14	14.30±0.92
ζ-Carotene	-	0.17±0.04	tr	0.46±0.28
Lycopene	0.58±0.08	43.90±1.93	0.04±0.00	42.40±1.36
β-carotene	0.48±0.02	2.58±0.11	0.44±0.22	3.51±0.58
Zeaxanthin	0.11±0.01	-	0.11±0.01	-
Violaxanthin	3.14±0.16	0.48±0.08	1.26±0.29	0.57±0.20
Lutein	0.52±0.04	0.01±0.01	0.45±0.02	-
Total carotenoids	**19.20±0.22**	**85.60±4.12**	**26.80±0.77**	**117.00±2.10**

tr < 0.05 µg/g FW

Ethylene production and expression of ethylene biosynthetic and receptors genes during cold storage of Marsh and Star Ruby grapefruit

Since ethylene has been described to play a role in CI development in grapefruit, emission of ethylene and transcriptional changes in biosynthetic (ACC synthase-*ACS1* and *ACS2* and ACC oxidase-*ACO*) and receptors (*ETR1, ETR2* and *ETR3*) genes were evaluated during storage of MSH and of NC (that developed CI) and C (tolerant to the disorder) SR fruit (Fig. 5). Ethylene production was similar in all fruit examined up to 25 days of cold storage (Fig. 5A). After that period, emission of the gas was higher in fruit developing CI than in tolerant C fruit. During 7 days of shelf-life at 20 ºC, ethylene production increased and was more than twice in CI-damaged fruit (MSH and NC SR) than in those tolerant to CI (C SR fruit, Fig. 5A).

The changes in the expression of ethylene biosynthetic genes during storage at 2 ºC were in agreement with the evolution of ethylene production. *ACS1*, *ACS2* and *ACO* transcripts markedly increased after storage at 2 ºC in the rind of fruit developing CI. The major increments were registered in the accumulation of *ACO* transcripts followed by *ACS1* and *ACS2* in MSH grapefruit which was several times higher than in the NC CI-sensitive SR (Fig. 5B). It is noticeable that in the red rind of CI-tolerant SR (C fruit), abundance of the *ACS1*, *ACS2* and *ACO* transcripts was negligible as compared with the rind of sensitive fruit, indicating that stimulation of ethylene biosynthetic genes is a chilling response (Fig. 5B).

The relative variation in the expression of *ETR* genes was lower than that of ethylene biosynthetic genes. In general, *ETR* genes were up-regulated during cold storage in the three types of fruit evaluated. Stimulation of *ETR1* and *ETR3* genes was in accordance with the severity of cold-induced damage, with a lower expression in the rind of C fruit. However, *ETR2* was more expressed in NC SR than in the white MSH (Fig. 5C). It is interesting to mention that a slight but significant increment in the expression of *ETR1* and *ETR3* in response to cold was detected in the rind of the CI-tolerant SR (C fruit), which may indicate a contribution of cold in the induction of these *ETR* genes (Fig. 5C).

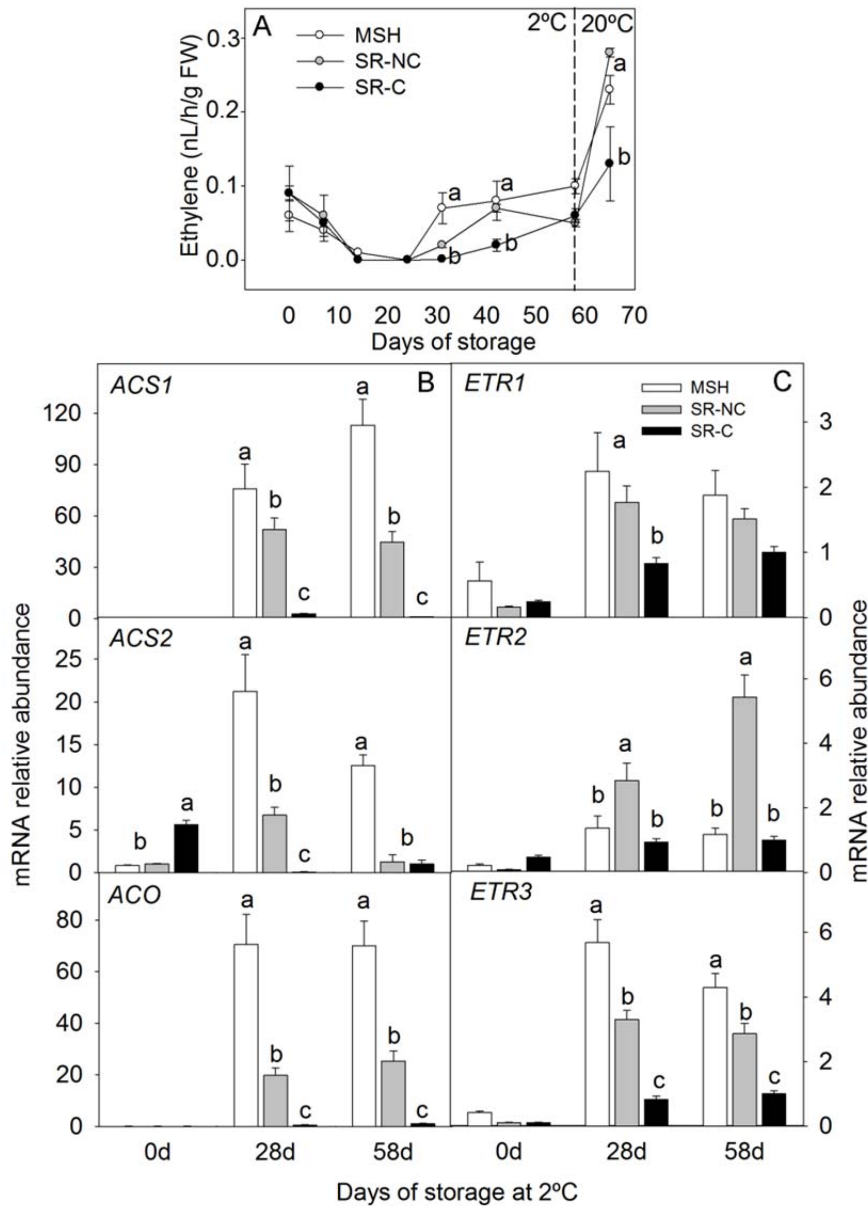

Figure 5. Ethylene production (nL/g FW/h) in Marsh (MSH) and non-covered (NC) and covered (C) Star Ruby (SR) grapefruit stored for up to 58 days and 7 days at 20 ºC (A). Accumulation of ethylene biosynthetic genes (*ACS1*, *ACS2*, *ACO*) (B) and ethylene receptors (*ETR1*, *ETR2* and *ETR3*) (C) in the flavedo of MSH and NC and C SR grapefruit stored 28 and 58 days at 2 ºC. Dotted lines show changes on storage temperature.*different letters indicate significant differences between means at each sampling date ($p<0.05$, Tukey test for ethylene and pair wise randomization test for gene expression). The absence of letters indicates no significant differences.

DISCUSSION

Citrus fruit quality is highly determined by external appearance, which directly influences marketability and consumer acceptance. Maintenance of high quality during transport, storage and extended postharvest life of citrus fruit is currently based on the use of low temperatures. However, some citrus species are highly sensitive to cold and develop CI symptoms in the rind. The white Marsh and the red Star Ruby grapefruit varieties are among the most cold-susceptible citrus varieties and develop CI both in the field and during postharvest cold storage (Schirra et al., 1998; Dou, 2005; Sapitnitskaya et al., 2006). CI symptoms in our study were apparent after 14 days storage at 2 ºC in MSH grapefruit and slightly later in SR (Fig. 1). These differences in the initiation and development of CI symptoms may be due to seasonal variations and also to varietal susceptibility to CI, but in general, chilling symptoms were similar to those previously observed in fruit of the same varieties growing in different countries and under distinct environmental conditions and cultural practices (McCollum and MacDonald, 1991; Schirra, 1992; 1998; Maul et al., 2008; 2011). However, comparison of chilling response in fruit of MSH and SR grapefruit revealed a striking difference between the two varieties; while chilling symptoms were distributed uniformly throughout the whole rind surface of MSH fruit, in SR grapefruit were restricted to the yellow areas and the red sections were almost completely absent of damage (Fig. 1). This particular and irregular distribution of CI symptoms was consistently observed in fruit of SR harvested from different orchards and also independently of the extension of the red blotches. It is remarkable that we also observed absence of CI in red zones of SR fruit harvested earlier on the season and when the surrounding rind was still greenish (data not shown). From these observations we concluded that the red zones of SR grapefruit showed a higher tolerance to CI during postharvest storage at low temperature.

The tolerance to CI in the red areas of SR was confirmed by ultrastructural TEM observations. Epidermal cells of red chilling-tolerant tissue presented a normal morphology with a defined membrane, an intact typical vacuole and well developed plastids (Fig. 2A). These structures are characteristic of healthy tissue and denoted absence of chilling damage after prolonged storage at 2 ºC. By contrast, yellow areas of fruit from both varieties stored for 58 days under the same temperature showed evident CI symptoms, characterized by membrane shrinkage and loss of cell integrity, and undefined organelles (Fig. 2B and C). Cell wall damage was also evidenced by constriction sites that became narrower compared to intact walls of healthy cells (Fig. 2). Similar ultrastructural changes have been previously described the rind of MSH, where low temperature induced damage of epidermal cells and several layers of the mesophyll cells below. An increment in lipid material inside the cytoplasm and the vacuole together with cytoplasm degradation and collapse were also described as deleterious damage in grapefruit under cold stress (Platt-Aloia et al., 1976). In our TEM observations, a clear separation of the internal membrane from the cell wall was shown in cells of rind-damaged grapefruit (Fig. 2B and C), a defect that has been also described in chilling-damaged eggplant and was triggered by the contraction of the cytoplasm and plasmatic membrane (Concellón et al., 2007). These morphological alterations in cell walls of cold-damaged tissue may be due to the changes in the composition of cell wall that would provoke weakness and increase its permeability, favoring then membrane damage and finally leading to cell death. The maintenance of cell wall structure and its components (mainly higher water soluble pectins and calcium bridges) are associated with the tolerance to CI induced

by high-temperature conditioning in the chilling-sensitive ‘Fortune’ mandarin (Holland et al., 2012), reinforcing the importance of cell wall composition on membrane integrity in the cold-induced damage. Absence of visible damage and weakness in cell wall of cells from red tissue of cold-stored SR grapefruit provides additional evidence of the resistance of this tissue to cold damage.

Changes in carotenoid content and composition are probably the most obvious differences between yellow chilling-sensitive and red chilling-tolerant rind tissue. It should be noted that the yellow tissue of the white MSH grapefruit was almost devoid of carotenoids (Table 1), as previously described (Alquezar et al., 2013), but the corresponding yellow tissue of SR have a high carotenoid content in which nearby 75% of the total was the colorless phytoene, and lycopene accounted for only 3% (Table 1). These differences in carotenoid content may probably be associated with the different susceptibility of both grapefruit varieties to CI, as MSH is recognized by a higher sensitivity to develop CI than red grapefruit (Dou, 2005) that were also evidenced in our study (Fig. 1). Despite the relatively high carotenoid content detected in the yellow rind of SR grapefruit, the tissue developed chilling damage that was almost indistinguishable to that occurring in the yellow rind of MSH. These results indicate that total carotenoid content appears not to be the main factor responsible for the induction of tolerance to CI in red zones of SR, being more likely related to the carotenoid composition and, specifically, to lycopene concentration. The content of this linear carotene was 14-times higher in the red than in yellow zones of SR grapefruit and it was completely absent in the rind of MSH (Table 1). Then, our working hypothesis was addressed to establish a potential relationship between lycopene content and tolerance to CI in red tissue of SR grapefruit. We reasoned that if by chemical manipulation of carotenoid biosynthesis, through application of CPTA, an inhibitor of β-lycopene cyclase enzyme (Coggins et al., 1970; Yokoyama et al., 1972), the lycopene content could be increased in MSH grapefruit, it would enhance the tolerance to CI. As a result, CPTA-treated MSH grapefruit developed an intense red coloration, and the rind accumulated 13-times more total carotenoids than non-treated fruit and a relative high content of lycopene (32 µg/g FW) which was absent in control ones (Table 2). As expected, this increment in lycopene content was associated with a significant delay in the initiation and a reduction of chilling damage in the chilling-sensitive MSH grapefruit (Fig. 3). It is interesting that despite there being different varieties, total carotenoids in CPTA-treated MSH fruit were lower than in yellow areas of SR (Table 1), but lycopene content was almost 20-times higher, suggesting the accumulation of lycopene above a threshold may be a key factor in the induction of tolerance to chilling.

In a second approach, we were able to significantly enhance chilling tolerance in SR grapefruit by fruit bagging. Light avoidance in SR grapefruit modifies expression of carotenoid biosynthetic genes and stimulated lycopene accumulation (Lado et al., unpublished results). By this environmental manipulation during fruit development, lycopene was increased as much as 40 µg/g FW, accounting between 36-52% of total carotenoids in the rind of SR fruit, which is a massive amount of lycopene and a unique feature among *Citrus* fruit (Rodrigo et al., 2013a). The concentration of linear carotenes, phytoene and phytofluene as well as of the cyclic carotenoid β-carotene, were also between 2 to 8 times higher in bagged than in non-bagged SR fruit (Table 3). Although these experimental strategies may provoke other unknown effects on fruit physiology, the changes on carotenoid concentration confirmed our rationale and reinforced the concept that the

increased tolerance to CI in red rind of grapefruit could be related to high carotene content, mainly lycopene.

Elevated lycopene content has been linked to lower CI incidence in tomato during cold storage (Whitaker, 1994) and chilling stress was described to reduce lycopene content in tomato (Rugkong et al., 2011), establishing then a possible linkage between these two factors. In citrus fruit, cold stress has been associated with an oxidative burst (Sala, 1998; Sala et al., 2000) and carotenoids, specially lycopene, display a powerful antioxidant activity (Krinsky, 1989; Aizawa et al., 2011) and ROS scavenging ability, then modulating redox signaling (Palozza et al., 2012). It is reasonable to assume that the tolerance to CI injury observed in the red areas of grapefruit could be due to an increased antioxidant capacity induced by the high lycopene concentration in that tissue. The experimental treatments designed to increase lycopene content also promoted accumulation of upstream linear carotenes, such as phytoene or phytofluene (Tables 2 and 3). Then, we cannot discard that altogether these increments in linear carotenes could participate in the induction of tolerance to CI in grapefruit. In this context, 'Pinalate', a sweet orange mutant with elevated amounts of phytoene, phytofluene and ζ-carotene, was more tolerant to CI than the ordinary sweet orange fruit (Alférez et al., 2005).

A partial contribution of β-carotene cannot be ruled out since this cyclic carotene also displays antioxidant activity (Aizawa et al., 2011) and its content was slightly enhanced in bagged fruit (Table 3). Moreover, the localization of β-carotene inside membranes could favor a higher fluidity during low temperature stress, promoting cold acclimation and therefore, favoring CI tolerance. Besides their well-known function as light collectors and photoprotectors, carotenoids are instrumental as structural components for the stabilization of the three-dimensional integrity of membranes under potentially harmful environmental conditions (Havaux, 1998).

Evidence from transgenic strategies reinforces the role of carotenoids as oxidative stress scavengers. Induction of high carotenoid accumulation in sweet potato *calli* (Kim;Kim;et al., 2013) or cultured cells (Kim et al., 2012) conferred resistance to salt-induced oxidative stress. The over-expression of different enzymes of carotenoid biosynthesis (phytoene synthase-*PSY* and lycopene cyclase-*βLCY*) in *Arabidopsis,* determined a higher accumulation of carotenoids and also an increased resistance to salt-mediated oxidative stress (Han et al., 2008; Chen et al., 2011). Furthermore, other powerful antioxidant molecules, such as anthocyanins, may protect fruit from postharvest stress conditions and pathogen infection (Zhang et al., 2013). Similarly, increasing ascorbic acid in tomato fruit was also associated with a higher chilling tolerance (El Airaj et al., 2013). Taken together, carotenoids and CI data in MSH and SR grapefruit suggest that the presence of higher levels of these antioxidants, mainly lycopene, could favor rind tolerance to oxidative stress induced by cold.

Analysis of the expression of ethylene biosynthetic and receptor genes in the rind of grapefruit varieties with contrasting susceptibility to CI has been useful to evaluate the metabolism of the hormone in the response of grapefruit to cold temperature. The implication of ethylene in fruit chilling stress is still controversial (Lafuente and Zacarías, 2006; Sevillano et al., 2009; Wang et al., 2013). Our results indicate that the ethylene biosynthesis genes analyzed *ACS1*, *ACS2* and *ACO* were clearly induced in relation to the chilling damage (Fig. 5B). These patterns of changes are in agreement with the evolution of ethylene production after 28 days of cold storage that was higher in the rind of fruit developing CI (MSH and non-covered SR) than in red-covered SR (Fig.

5A). *ACS1* isoform showed higher activation than *ACS2* in damaged tissues, suggesting an important role of this isoform in the response of grapefruit to chilling damage. Maul et al. (2008) also found induction of *ACS* and *ACO* by chilling and that each gene was differentially affected by heat conditioning. Our results showed that the three genes of the ethylene biosynthesis analyzed were induced in relation to rind damage which indicates that these genes may be good markers of CI and that their induction during postharvest storage of grapefruit is more likely related to CI than to cold response.

Regarding ethylene perception, expression of three members of the *ETR* gene family revealed a differential and complex regulation of each member, not only by chilling but also by cold. Similar to ethylene biosynthetic genes, *ETR1* and *ETR3* were up-regulated in a chilling-dependent manner (Fig. 5C). However, in the red rind of CI-tolerant SR, accumulation of both transcripts was also increased, although to a lower level than in yellow CI-sensitive tissue. This indicates that the expression of *ETR1* and *ETR3* is stimulated in grapefruit by both factors, chilling injury and cold stress. To our knowledge, these are the first results showing changes in ethylene receptor genes during storage of citrus fruit at low temperature and linking ethylene perception with cold stress and chilling damage. Moreover, ethylene receptor genes are up-regulated by exogenous ethylene in other citrus (Katz et al., 2004; John-Karuppiah and Burns, 2010; Alós et al., 2014). It is then plausible that the induction of ethylene biosynthetic genes and ethylene production during storage of chilling-sensitive fruit at low temperatures may stimulate *ETR* gene expression. Whether ETRs may function during cold storage of citrus fruit as negative regulators of ethylene signaling or stimulating other ethylene-related responses remains to be elucidated (Binder, 2008; Wang et al., 2013).

In summary, our results reveal a positive relationship between carotenes content, mainly lycopene, and chilling tolerance during storage of grapefruit at low temperature. The potential protective role of lycopene in CI has been explored by chemical and environmental stimulation of the content of this carotene in the rind of grapefruit. The antioxidant properties of lycopene are suggested to mediate the tolerance of the red zones to CI. Moreover, expression of ethylene biosynthetic genes appears to be induced in a chilling-dependent manner. However, chilling and also cold participate in the induction of the ethylene receptors genes *ETR1* and *ETR3*.

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3.2. CAPÍTULO II.

Analysis of ethylene biosynthesis and perception during postharvest cold storage of Marsh and Star Ruby grapefruits

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Abstract

Grapefruits are among the citrus species more sensitive to cold and develop chilling injury symptoms during prolonged postharvest storage at temperatures lower than 8-10 ºC. The plant hormone ethylene has been described either to protect or potentiate CI development in citrus whereas little is known about transcriptional regulation of ethylene biosynthesis, perception and response during cold storage and how the hormone is regulating its own perception and signaling cascade. Then, the objective of the present study was to explore the transcriptional changes in the expression of ethylene biosynthesis, receptors and response genes during cold storage of the white 'Marsh' and the red 'Star Ruby' grapefruits. The effect of the ethylene action inhibitor, 1-MCP, was evaluated to investigate the involvement of ethylene in the regulation of the genes of its own biosynthesis and perception pathway. Ethylene production was very low at the harvest time in fruits of both varieties and experienced only minor changes during storage. By contrast, inhibition of ethylene perception by 1-MCP markedly induced ethylene production, and this increment was highly stimulated during shelf-life at 20 ºC, as well as transcription of *ACS* and *ACO*. These results support the auto-inhibitory regulation of ethylene in grapefruits that acts mainly at the transcriptional level of *ACS* and *ACO* genes. Moreover, *ETR1* and *ETR3* were induced by cold while no clear role of ethylene was observed in the induction of *ETR*s. However, ethylene appears to be implicated in the transcriptional regulation of *ERFs* both under cold storage and shelf life.

INTRODUCTION

Storage at low temperatures is one of the most practical and widely used technologies to maintain quality of fruit and vegetables during postharvest transportation and storage. However, fruit of some citrus species are susceptible to develop chilling injury (CI) symptoms when temperatures drop below 8-10 ºC. Fruits of the different grapefruit varieties (*Citrus paradisi* Macf.) and lemon (*Citrus limon*) are among the most sensitive to develop CI (Schirra, 1992; Lafuente and Zacarias, 2006). CI symptoms in grapefruits are initially manifested as small brown pits on the fruit surface that progressively increase in size forming brown clusters of depressed areas of different shape, that may affect large surface of the flavedo in prolonged cold storage periods (El-Otmani et al., 2011). The sensitivity to CI is variable and depends on the variety and other endogenous and environmental factors. Among relevant grapefruit commercial varieties, the white 'Marsh Seedless' has been described to be extremely sensitive to cold-induced damage, followed by the red-fleshed 'Star Ruby' and 'Red Blush' varieties (Schirra et al., 1998). In general, grapefruit harvested early and later on the season are more sensitive to CI than mid-season fruits (Dou, 2005a; Lafuente and Zacarías, 2006).

Despite the susceptibility to CI of grapefruits and other citrus cultivars, storage at temperatures between 1-2 ºC for different periods are required for quarantine treatment to export citrus fruits to several countries, as Japan or USA. Therefore, different methods have been evaluated and proposed to alleviate the severity of chilling damage in sensitive citrus varieties. One of the most studied is the application of high temperatures (37-50 ºC) by curing or hot water dips before cold storage, which showed a significant reduction in CI incidence in different sensitive varieties like 'Fortune' mandarin (Sala and Lafuente, 1999; Lafuente et al., 2011), lemon, grapefruits (Rodov et al., 1995) and Satsuma mandarins (Ghasemnezhad et al., 2008). As an alternative, application of mild temperatures around 16 ºC for one week before storage showed a reduction in CI incidence in different grapefruits (Sapitnitskaya et al., 2006; Maul et al., 2008; Maul et al., 2011). Similarly, intermittent warming (3 weeks at 3 ºC followed by 2 weeks at 15 ºC) has been described to be highly effective on inducing chilling tolerance in cold-stored fruits (Schirra et al., 1999). However, nowadays the application of high temperatures is not an attractive method to be applied in commercial conditions mainly due to technological limitations and the high risk to provoke peel damage by the extreme high temperatures (Ghasemnezhad et al., 2008).

The physiological and molecular bases related to the sensitivity of citrus fruits to CI and also to the heat-induced tolerance to CI have been extensively studied over the years. These studies are critical to understand how these processes and treatments are genetically controlled and to provide the basis for further biotechnological manipulation and development of newly successful strategies (Lafuente et al., 2004; Maul et al., 2008). The phytohormone ethylene has been implicated in the plants responses to different biotic and abiotic conditions (Wang et al., 2013). However, the involvement of ethylene in the response of horticultural commodities to cold stress is controversial, and results indicating either a protective role or causing deleterious effects on CI have been reported (Sevillano et al., 2009). In avocado and plums, application of exogenous ethylene accelerated CI symptoms (Candan et al., 2008), while in nectarines alleviated its development (Zhou et al., 2001). In citrus fruit, ethylene has been related to many stress responses and also was described to be induced under chilling conditions in 'Marsh' grapefruits stored at 5

ºC, even before the appearance of CI symptoms (McCollum et al., 1991). In fruits of the hybrid mandarin 'Fortune', which is very sensitive to develop CI (Lafuente and Zacarías, 2006), exogenous application of ethylene before cold storage led to an increment in the susceptibility to this disorder (Lafuente et al., 2001; Gosalbes et al., 2004). However, a continuous application of the gas at low concentrations during storage in the same variety induced tolerance to chilling (Lafuente et al., 2004), suggesting that ethylene is part of the protective defense mechanisms to maintain fruit natural resistance against various environmental stresses (Porat et al., 1999; Marcos et al., 2005). The response to ethylene appears to be variable and dependent of the variety or the hormone dose applied, as continuous application of different ethylene concentrations during storage of 'Tahitian' lime, 'Emperor' mandarin, 'Marsh' grapefruit and 'Valencia' oranges at 0 ºC resulted in a higher incidence of CI that was proportional to the ethylene dose (Yuen et al., 1995). Moreover, the incidence of CI in 'Marsh' grapefruit was also positively related to the duration of the ethylene treatment before storage (Hatton and Cubbedge, 1981). Similarly, data from the application of the inhibitor of ethylene perception, 1-MCP, reinforces the notion that the role of ethylene in CI may depend on the citrus variety considered. Thus, 1-MCP treatment to 'Nova' and 'Ortanique' varieties significantly reduced CI symptoms after 60 days at 1 ºC (Salvador et al., 2006) as also occurred in 'Murcott' (Edagi et al., 2010) and 'Fallglo' tangerine (Dou et al., 2005b). By contrast, fruits of the chilling-sensitive 'Fortune' mandarin treated with 1-MCP developed faster and higher CI during storage at 2 ºC, suggesting that in this variety as well as in oranges ethylene is also part of the defense mechanism developed to cope cold stress (Porat et al., 1999; Lafuente et al., 2001; Marcos et al., 2005).

The transcriptional regulation of ethylene biosynthesis and perception has been also described to be affected by chilling conditions in different fruits. Early studies in 'Passe-Crassane' pears, a cultivar that require exposition to low temperature to ripen, demonstrated an accumulation of *ACS* and *ACO* transcripts as well as of ACO protein during postharvest chilling treatment. After transference to shelf-life conditions, ACO protein was able to oxidize ACC and then to increase ethylene production, stimulating pear fruit ripening (Lelièvre et al., 1997). Ethylene biosynthetic genes (ACC synthase-*ACS* and ACC oxidase-*ACO*) as well as ethylene receptors (*ETRs*) transcripts were induced during cold stress in the field in climacteric avocado fruit (Hershkovitz et al., 2009). In tomato fruit, *ACS* gene expression showed a transient increase after one week of cold storage, while *ETR* expression pattern was very variable and strictly depended on the gene isoform evaluated (Rugkong et al., 2011). A marked induction in *ETR1* expression was registered in loquat fruit stored at low stressful temperatures, being directly correlated with CI development, however other isoforms showed different patterns of expression in response to cold stress (Wang et al., 2010).

In citrus fruit, previous studies have evidenced an increment in ethylene production during postharvest storage at low temperatures (Cooper et al., 1969; McCollum and McDonald, 1991; Schirra, 1992, Lafuente et al., 2001). Regulation of this cold-induced ethylene production is still not well understood, and it appears that the response of the different varieties may be different and also dependent on the susceptibility to CI. In the peel of orange fruits Wong et al. (1999) observed the transcript accumulation of one *ACS* isoform that appears to be dependent on the time of storage under cold conditions. Large-scale analysis of gene expression in cold stored and heat-conditioning

grapefruits has revealed accumulation of *ACO* gene transcript (Maul et al., 2008; Zhu et al., 2011). In the chilling sensitive 'Fortune' mandarin, it has been observed an increment in ethylene production during storage at 2 ºC concurrently to the development of CI symptoms (Lafuente et al., 2001). Moreover, in the peel of this mandarin *ACS2* and *ACO* transcripts, as well as ACO protein and the ethylene metabolic precursor ACC, were accumulated during the chilling period. Therefore, upon transference to shelf-life at 20 ºC there was an overstimulation of ethylene production at the expenses of the pre-formed ACO protein and the substrate ACC (Zacarías et al., 2003). This regulation of ethylene biosynthesis under cold stress may explain the rapid and massive increase in ethylene production usually detected in fruits upon transference to shelf-life, at temperatures near the optimum for enzyme activity. However, our understanding about how ethylene biosynthesis is regulated by cold stress and how these changes are coordinated with ethylene perception and responses to these postharvest conditions in citrus fruits are still unknown. Then, the objective of the present study was to explore the transcriptional changes in the expression of ethylene biosynthesis, receptors and response genes during cold storage of the white 'Marsh' and the red 'Star Ruby' grapefruits. The effect of the ethylene action inhibitor, 1-MCP, was also evaluated to ascertain the involvement of ethylene in the regulation of the genes of its biosynthesis and perception pathway.

MATERIALS AND METHODS

Plant material, treatments and storage conditions

Fruit of grapefruit (*Citrus paradisi*) cv. 'Marsh' (MSH) and cv. 'Star Ruby' (SR) from adult trees cultivated under commercial conditions in an orchard located in Moncada, Valencia (Spain), were harvested in January at full maturity. Fruits were delivered to the laboratory, inspected for free of injuries or defects, selected by uniformity and divided in replicated samples. Replicate lots of fruits were treated with 1 µL/L of the inhibitor of ethylene action 1-MCP, as described in Lafuente et al. (2001), or remained in a continuous air flow for 16 hours at 20 ºC and 90% RH. After the treatment, fruits were ventilated and stored at 2 ºC (chilling temperature) or 12 ºC (non-chilling control temperature) and 80-85% RH for up to 58 days. 1-MCP treated fruits were stored only at 2 ºC. After 58 days of storage at 2 ºC, replicate samples of fruits were transferred for 7 days at 20 ºC to simulate a shelf life period. During postharvest storage and shelf life, CI incidence and ethylene production were determined in cold stored and control fruit. At each sampling date (0, 24 and 58 days, and 58 days +7 days at 20 ºC) flavedo tissue was excised from the whole fruit, frozen in liquid nitrogen, ground to a fine powder and stored at -80 ºC until analysis.

Estimation of chilling injury index

Fruit were inspected for the intensity and extension of chilling damage and rated on a scale from 0 (no damage) to 3 (severe damage) (Schirra et al., 1998). Results were expressed as peel damage index that was calculated according to the formula:

Σ (Peel damage scale (0-3) x number of fruit within each class)/total number of fruit.

Three replicates of 10 fruits per treatment, temperature and grapefruit variety were used for CI evaluation using a complete randomized design.

Determination of ethylene production

Ethylene production was determined by incubating 3 replicates of 3 fruit each into 4 L flasks that were hermetically sealed and maintained at the storage temperature. After 3 h of incubation, 1 mL air samples from the headspace of the flasks was withdrawal with a hypodermic syringe and injected into a gas chromatograph (Perkin Elmer Autosample) equipped with a flame ionization detector and an activated alumina column. Nitrogen was used as carrier gas and the temperature of the column was maintained at 140 ºC. Ethylene production values represent the mean of three replicates and for statistical significance an ANOVA test was performed and Tukey test applied for mean separation ($p \leq 0.05$).

Quantitative Real Time-PCR

Total RNA was isolated from the flavedo of fruit at each harvesting date, using RNeasy Plant Mini Kit (Qiagen) and subsequently treated with DNase (DNA free, DNase treatment & removal, Ambion). Procedure followed for relative gene expression quantification and primers used for gene amplification as well as software details are described in Alós et al. (2014). Normalization was performed using the expression levels of the *Actin* gene as reported in Alós et al. (2013). For each gene a relative value of 1 was assigned to the expression determined in the flavedo of SR grapefruit at harvest time. Results were the average of 4 independent replicates.

RESULTS AND DISCUSSION

To understand the regulation of ethylene biosynthesis and perception during cold storage of grapefruits, mature fruits of the white 'Marsh' and the red 'Star Ruby', both very sensitivity to develop CI (El-Otmani et al., 2011), were stored at 2 ºC for up two month, followed by a simulation of shelf-life period for 7 days at 20 ºC. Evolution of ethylene production and CI index was followed in fruits of both varieties (Fig. 1). To understand the involvement of ethylene in its own synthesis and the potential effect on CI development, grapefruits were pre-treated with the inhibitor of ethylene action, 1-MCP (Watkins, 2006) and then stored at 2 ºC.

In MSH and SR grapefruits CI symptoms appeared after 14 days of storage at 2 ºC in both 1-MCP treated and control fruit, while fruits stored at 12 ºC remained undamaged during the whole storage period and did not show any symptoms of CI. Symptoms were characterized by brown depressed pitting areas that progressively became dark-brown lesions covering larger surface of the fruits as advanced the cold storage period (Fig. 1A). CI incidence increased progressively during the storage, reaching values around 2 in MSH and 1.5 in SR variety after 2 months, and it did not increase substantially after the simulation of the shelf-life period. A pre-treatment with the ethylene action inhibitor, 1-MCP, reduced CI damage in SR fruit, which was less prone to develop CI. However, this effect of 1-MCP was not corroborated in MSH variety (Fig. 1) and neither in different seasons (data not shown). It is interesting to remark that in fruits of the red SR grapefruit, CI symptoms were restricted only to the yellow areas of the peel, and those of red coloration were virtually free of pitting or any other CI symptoms (data not shown). Many of the fruits of SR

grapefruit developed red areas in the peel although of variable extension and intensity, and this may be the reason of the lower CI index in fruits of this grapefruit in comparison with those of MSH (Fig. 1A-B). This tolerance of red-colored areas of the peel of SR grapefruit to CI has been the subject of parallel studies and investigations (Lado et al., unpublished results).

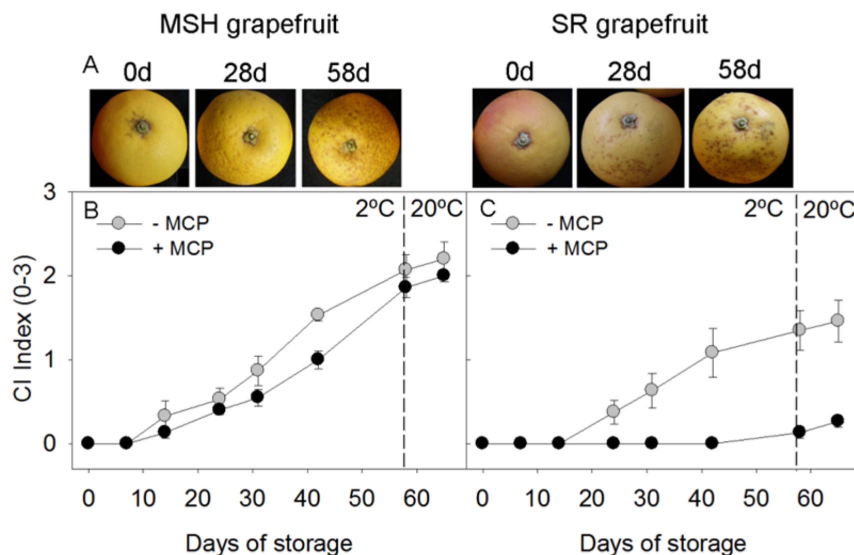

Figure 1. Chilling injury symptoms in the peel of MSH and SR grapefruit after 28 and 58 days of storage at 2 ºC (A) and evolution of CI (0-3) during storage at 2 ºC for up to 58 days and after 7 days of shelf-life at 20 ºC in fruits of MSH (B) and SR (C) grapefruits treated or non-treated with 1-MCP.

Ethylene production was very low at the harvest time in fruits of both varieties and experienced only minor changes during storage of non-treated control fruit (Fig. 2). By contrast, inhibition of ethylene perception by 1-MCP markedly modified the rate of ethylene production in fruits of both varieties. After 4 weeks in MSH or 2 weeks storage in SR, 1-MCP-treated fruits produced more than 10 times ethylene compared with control fruits (inset panels Fig. 2). This overstimulation of ethylene production by 1-MCP was also manifested after rewarming. Then, only 1 day after transfer from 2 to 20 ºC, a massive burst of ethylene production was measured reaching values as higher as 12-15 nL/h/g (Fig. 2). This massive increment on ethylene production in 1-MCP-treated fruit is consistent with previous results in citrus fruits (Mullins et al., 2000; Lafuente et al., 2001; Katz et al., 2004; Marcos et al., 2005) and reinforced the motion of a negative feedback regulation by ethylene of its own biosynthesis. This mechanism was initially described in the wound-induced ethylene production in the peel of citrus fruits (Riov and Yang, 1982) and has been also observed in pathogen and cold-induced ethylene biogenesis (Lafuente et al., 2001; Marcos et al., 2005). Auto inhibition of stress-induced ethylene production implicates that ethylene represses the biochemical and molecular processes controlling its own biosynthesis and under absence of ethylene action, as by 1-MCP treatment, the inhibition is lost and ethylene production is over-stimulated (El-Otmani et al., 2011), as was observed in cold-stored grapefruits (Fig. 2).

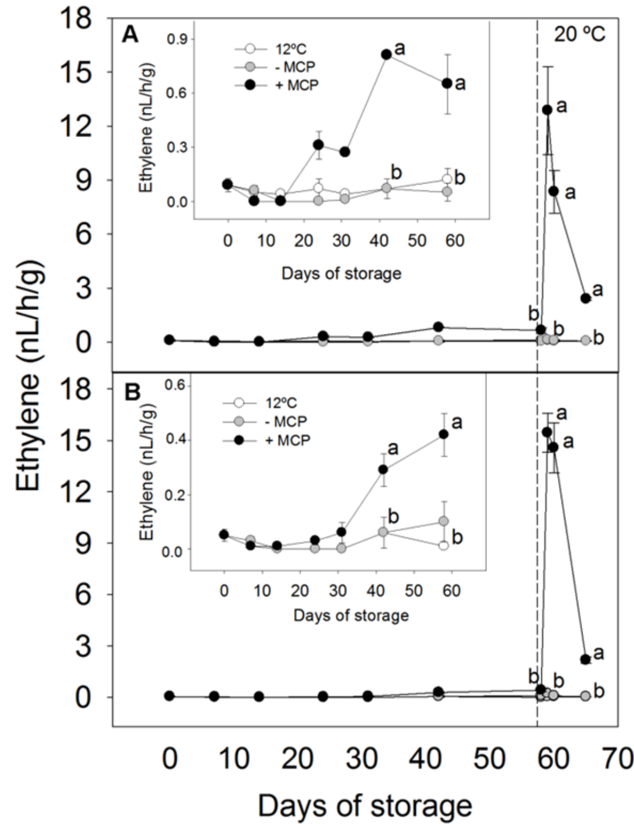

Figure 2. Ethylene production (nL/h/g) in MSH (A) and SR (B) grapefruits treated or non-treated with 1-MCP and stored for up to 58 days at 2 ºC and after 7 days of shelf-life at 20 ºC. Control fruit was stored at 12 ºC and transferred to 20 ºC for shelf-life simulation. Inserted panel show ethylene production in MCP-treated and non-treated fruits stored at 2 ºC and 12 ºC for up to 58 days. Different letters indicate significant differences between treatments at each date ($p \leq 0.05$).

Then, a positive relation between damage and ethylene emission could be suggested, as observed previously in cold-stored 'Fortune' mandarin (Zacarías et al., 2003). The involvement of ethylene in the development of CI is controversial and depends on the plant species since ethylene may play a preventive (Zhou et al., 2001; Dong et al., 2001) or inductive role (Dou et al., 2005b; Edagi et al., 2010; Orihuel-Iranzo et al., 2010). In citrus fruits there is also controversial the involvement of ethylene in CI development and if it is a cause or a consequence of chilling damage is still unclear. In 'Marsh' grapefruits and 'Fortune' mandarin the chilling-induced ethylene production was coincident with the initiation of CI symptoms (McCollum et al., 1991) but ethylene not always is able to mitigate the chilling-induced damage (Porat et al., 1999; Lafuente et al., 2001; Gosalbes et al., 2004). Moreover, experiments with 1-MCP did not show consistent results on CI development in fruits of different citrus varieties, and protective (Salvador et al., 2006) and negative effects (Lafuente et al., 2001) have been reported. Under other stress conditions, as pathogen infection, it has been observed that disruption of ethylene perception enhanced deleterious effects, suggesting that ethylene may be part of the protective role of the fruits to cope stress conditions (Porat et al., 1999; Marcos et al., 2005). The discrepancy on the relationship between ethylene and cold stress observed among fruits of different citrus varieties, suggests that

the response may be dependent on the variety and also on the particular sensitivity to CI, among other factors (Watkins, 2006).

To further understand the regulation of ethylene biosynthesis and perception during grapefruits cold storage, the expression of ethylene biosynthesis (ACC synthase-*ACS1* and *ACS2* and ACC oxidase-*ACO*), receptors (*ETR1*, *ETR2* and *ETR3*) and response factors (*ERF1* and *ERF2*) genes was analysed in the peel of fruits stored at 2 ºC and after shelf life at 20 ºC. Results of transcriptional analysis of these genes was virtually the same in both grapefruit varieties, indicating a similar regulation of ethylene metabolism, and therefore, and to avoid repetitive data, only results on Marsh genotype are thoroughly described (Figs. 3 to 5).

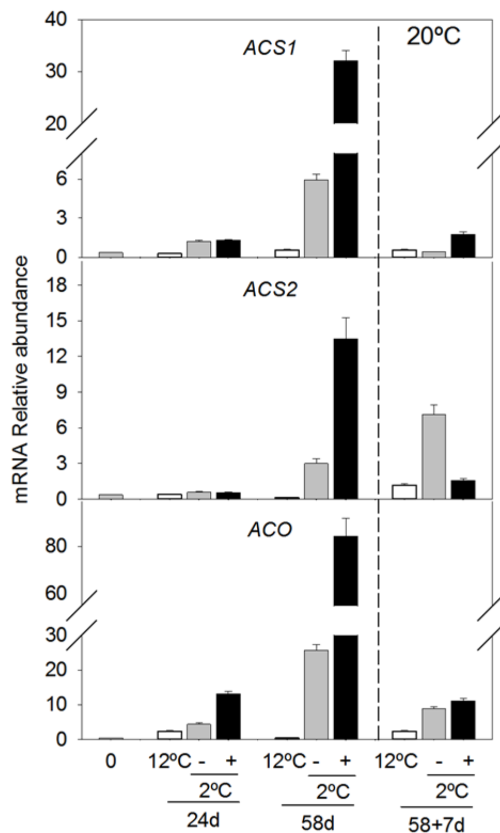

Figure 3. Expression of ethylene biosynthetic genes ACC synthase-ACS1 and ACS2 and ACC oxidase-ACO in the peel of MSH grapefruit treated (+) and non-treated (-) with 1-MCP and stored for 24 and 58 days at 2 ºC and after 7 days of shelf-life at 20 ºC. Control fruit was stored at 12 ºC and transferred to 20 ºC for shelf-life simulation.

Cold storage markedly induced the expression of the ethylene biosynthetic genes *ACS1, ACS2* and *ACO* which was especially overstimulated in the peel of 1-MCP pre-treated fruit, showing that the negative feedback regulation of the cold-induced ethylene production acts at transcriptional level (Fig. 3). It is interesting to mention that although both *ACS1* and *ACS2* transcripts were cold induced, the increment of the former was higher. In fruits of other citrus varieties exposed to different stressful conditions, differential accumulation of both *ACS* transcripts was observed, indicating a tissue or stress-specific response (Zacarías et al., 2003, Katz et al., 2004;

Marcos et al., 2005). Despite the transcription of *ACS* and *ACO* genes was also induced in response to cold (although with a different extent) this increment was not directly reflected on ethylene production during cold storage (Fig. 2). This effect could be explained by the partial inactivation of ACO enzyme activity under low temperatures (Lederman et al., 1997), a limitation that was clearly removed during shelf life at 20 ºC (Fig. 2). Thus, after fruit rewarming the enzymes of ethylene production may reach a temperature near optimum for activity, and therefore, ethylene production would be triggered at expenses of the metabolites (ACC) accumulated during the exposition to cold stress, similarly to that observed in other citrus cultivars (Zacarías et al., 2003). Moreover, after stress removal, *ACS1* and *ACO* genes expression declined (Fig. 3), indicating that cold directly triggers transcription of the ethylene biosynthetic genes. A similar induction of *ACS* and *ACO* genes during cold storage has been also described in MSH grapefruit stored under cold conditions (Maul et al., 2008; Maul et al., 2011). *ACS1* and *ACS2* genes were also stimulated in oranges stored at 4 ºC and drastically declined after fruit removal to 25 ºC, coinciding with a marked increment on ethylene production at higher temperature (Wong et al., 1999). It is worth mentioning that the overstimulation of *ACS1, ACS2* and *ACO* transcripts by 1-MCP is in agreement with the increment of ethylene production in 1-MCP-treated fruits exposed to 2 ºC. Although this stimulation of ethylene production was significant respect to non 1-MCP-treated fruits, it was much lower to the increment registered after rewarming that reached ethylene production levels as high as 15 nL/h/g (Fig. 2). Interestingly, after transference to 20 ºC, *ACS* and *ACO* transcripts abundance declined, indicating that cold is likely the stimuli inducing the expression of these genes and that ethylene is a negative regulator of its own biosynthesis in grapefruits.

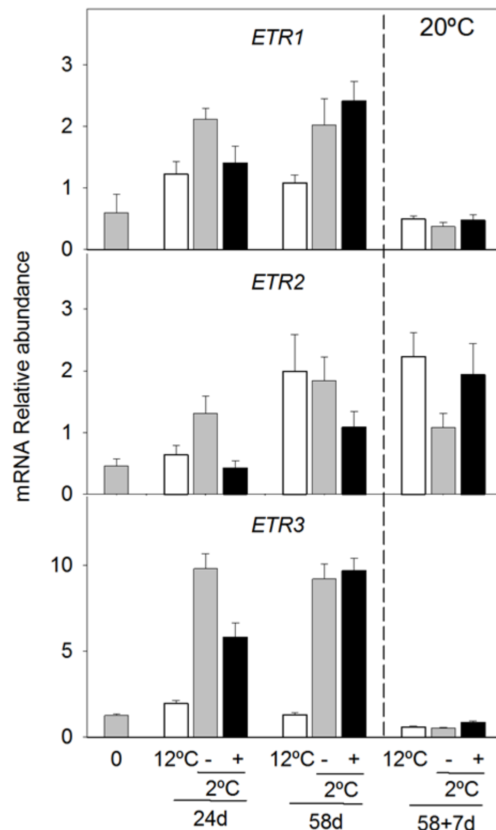

Figure 4. Expression of ethylene receptor genes ETR1, ETR2 and ETR3 in the peel of MSH grapefruit treated (+) and non-treated (-) with 1-MCP and stored for 24 and 58 days at 2 ºC and after 7 days of shelf-life at 20 ºC. Control fruit was stored at 12 ºC and transferred to 20 ºC for shelf-life simulation.

To analyse the effect of cold or chilling in the regulation of ethylene perception and signaling, the expression of three members of the ethylene receptor gene family, *ETR*, were measured during cold storage and shelf-life. Results of Figure 4 indicated that each *ETR* gene member analysed was differentially and independently induced by cold and/or ethylene. The three *ETR* genes were early up-regulated by cold, especially *ETR3*, since its relative expression level was higher in cold stored fruits compared to that in fruits exposed to non-chilling temperatures. The stimulation of *ETR1* and *ETR3* expression by low temperature was maintained after 58 days, but this did not occur with *ETR2*. Inhibition of ethylene action by 1-MCP produced variable effects on the expression of the three *ETR* genes since it reduced the cold-induced expression after 24 days but not after a more prolonged cold storage (Fig. 4). After fruit rewarming, expression of both *ETR1* and *ETR3* genes was substantially reduced, whereas that of *ETR2* remained with minor modifications. Collectively, these results indicated that *ETR3* gene was the most responsive to variations in storage temperature and that this gene and *ETR1* appears to be cold-induced. The involvement of ethylene in the regulation of *ETR* genes is complex with an apparent repression of the cold-induced stimulation in grapefruits (Fig. 4). Ethylene signaling cascade is composed by a interrelated network of different regulatory steps (Wang et al., 2013; Gapper et al., 2013) where the first responsible mechanism for the sensing of the hormone are ETRs, which are a complex family of isoforms varying in number in the different species. Five isoforms of receptors have been

described in *Arabidopsis* while six different elements were reported to exist in tomato fruit (Binder, 2008). In citrus fruit, three different isoforms for the ETR family of ethylene receptors have been described (Katz et al., 2004; Distefano et al., 2009; John-Karuppiah and Burns, 2010) and some of them, mainly *ETR2,* was induced by exogenous ethylene application during color change (Alós et al., 2013). Moreover, a positive correlation between transcript levels of ethylene receptors and ethylene sensitivity was suggested in citrus (Alós et al., 2013) and in apple fruits (Yang et al., 2013). These changes in the expression of ethylene receptors may be relevant to determine tissue sensitivity to the hormone, since ethylene action is thought to be negatively regulated by ethylene receptors (Gapper et al., 2013) and a high receptors level would repress ethylene response while low amount would enhance ethylene sensitivity (Agarwal et al., 2012). However, this process is very complex and experimental evidences not always correlated with the predicted model. In apple fruits, for example, the level of ethylene receptor genes increased with stimulation of ethylene response and natural ripening (Tatsuki et al., 2009; Yang et al., 2013). Our results are the first evidences of the involvement of the *ETR* gene family of ethylene receptors in the citrus fruits response to cold storage.

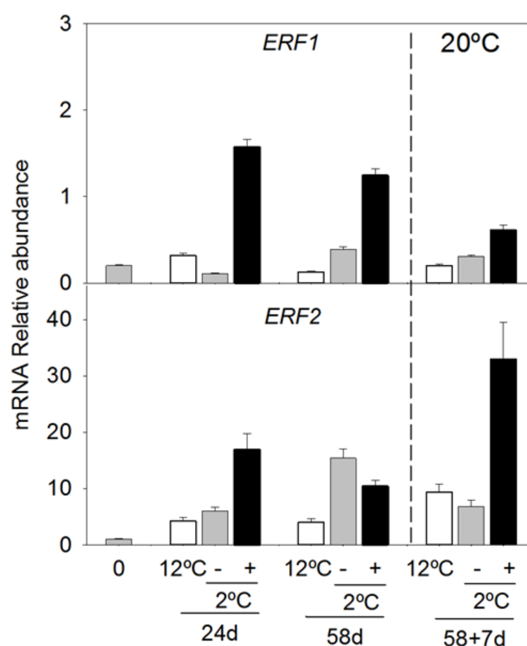

Figure 5. Expression of ethylene response factors genes ERF1 and ERF2 in the peel of MSH grapefruit treated (+) and non-treated (-) with 1-MCP and stored for 24 and 58 days at 2 ºC and after 7 days of shelf-life at 20 ºC. Control fruit was stored at 12 ºC and transferred to 20 ºC for shelf-life simulation.

Following downstream elements in the ethylene signaling cascade, ethylene response factors (ERFs) have been described as negative regulators of ethylene responses and essential modulators of fruit ripening and stress responses (Gapper et al., 2013; Wang et al., 2013). *ERF*s exhibited variable regulation by cold and 1-MCP treatments in grapefruits. The expression of *ERF1* was markedly stimulated by 1-MCP but not by low temperature (Fig. 5). On the other hand, *ERF2*

transcription was stimulated by cold and 1-MCP treatment both during low temperature storage and shelf life at 20 ºC (Fig. 5). These results suggest that these two ERF factors, especially *ERF2,* may be involved in the cascade of events induced during cold stress in grapefruits and that are negatively regulated by ethylene, since 1-MCP overstimulated their expression (Fig. 5). Similarly, it has been described that both *ERF*s are negatively regulated by ethylene in the peel of 'Comune' mandarin (Alós et al., 2013) while another group of ERFs (C-repeated binding factor-CBFs) have been described to be induced under cold and to display a relevant role in cold hardiness in citrus fruit and the close related *Poncirus* (Champ et al., 2007).

CONCLUSIONS

Collectively, from the results of the current study it could be concluded that cold markedly stimulates transcription of ethylene biosynthetic genes (*ACS* and *ACO* genes) and to a lesser extent, transcript abundance of a specific isoform of the ethylene receptor *ETR3*, and also of the ethylene response factor *ERF2*. Pre-treatment of grapefruit with the inhibitor of ethylene action, 1-MCP, before storage at 2 ºC induced a significant increase in ethylene production during cold storage that became massive after simulation of shelf-life at 20 ºC, indicating a negative feedback control by ethylene of its own biosynthesis. No clear effect of 1-MCP was observed in the induction of *ETR* gene expression. However, ethylene appears to be implicated in the transcriptional regulation of *ERFs* both under cold storage and during shelf life.

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3.3. CAPÍTULO III.

Implication of the antioxidant system in the tolerance to chilling injury in the red peel of grapefruit

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Abstract

Chilling injury (CI) is one of the most deleterious postharvest problems affecting external quality and marketability of fruit and vegetables. Fruit of the different Citrus species and varieties are sensitive to develop CI symptoms when stored below 10 ºC, being grapefruit among the cultivars especially prone to develop the disorder. In a previous study (Lado et al., 2015a) we demonstrated that red zones of grapefruit peel, containing high lycopene concentration, were substantially more tolerant to CI than yellow zones of both the yellow Marsh and the red Star Ruby varieties. Taking into account the powerful antioxidant capacity of lycopene, in the current work we hypothesized that the tolerance to CI in the lycopene-accumulating rind of grapefruit may be due to an enhancement of the enzymatic and/or non-enzymatic antioxidant systems. Then, we have evaluate total antioxidant capacity, content of the antioxidant metabolites GSH and AsA, and activity and gene expression of the antioxidant enzymes GR (glutathione reductase), ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) in the peel of Star Ruby grapefruit with contrasting tolerance to CI during storage at 2 ºC for up to 58 days followed by 7 days at 20 ºC. Fruit of Star Ruby grapefruit exposed to standard environmental conditions during the growing season developed yellow peel coloration and were sensitive to CI after cold storage. By contrast, fruit that were covered under filed conditions developed an intense red coloration and were tolerant to CI upon subsequent cold storage. The peel of CI-tolerant fruit exhibited a lower lipid peroxidation level (MDA content). Concentration of hydrogen peroxide was similar after 3 weeks storage, when the differences in chilling damage between sensitive an tolerant fruit were noticeable, suggesting that the increase in H_2O_2 is a response of the flavedo cells to cold stress not necessarily related to the development of CI. Moreover, tolerance to CI was not associated with an enhancement of neither total antioxidant capacity nor glutathione and AsA contents, indicating that such antioxidant responses may be cold-mediated and not directly linked to chilling tolerance. Analysis of singlet oxygen scavenging capacity, by the SOAC assay, revealed a considerably higher activity in the lycopene-accumulating peel than in the yellow peel at the harvest time and throughout the whole cold storage and shelf-life period. Enzymatic activity and gene expression analysis of GR, APX and SOD did not reveal the involvement of these antioxidant enzymes in the protection to CI. However, a high CAT activity was detected in the peel of CI-tolerant fruit, although this difference was not paralleled with changes in the expression levels of *CAT1* and *CAT2* genes. It is then concluded that the boost in singlet oxygen scavenging capacity is likely the main factor responsible of the tolerance to CI in the lycopene-accumulating peel of grapefruit.

INTRODUCTION

Cold storage is the most common and worldwide used storage technology to extend postharvest life of fruit and vegetables. However, fruit of subtropical origin are sensitive to cold storage, developing a myriad of symptoms and lesions generically referred to as chilling injury (CI). Among citrus fruit, grapefruit (*Citrus paradisi* Macf.) are highly susceptible to develop chilling injury when temperatures drop below 8-10 ºC (Schirra, 1992; Schirra et al., 1998; Dou, 2005). CI lesions in grapefruit are manifested as sunken brown spots of different shape and size restricted to the flavedo tissue and the susceptibility to chilling depends also on the variety and growing conditions, among other factors (Schirra, 1992; Schirra et al., 1998; Dou, 2005).

Alterations of cell membranes structure and stability are thought to be a primary response of plant cells to cold stress. Moreover, changes in lipid composition, including lipid peroxidation, an increase in the index of fatty acid saturation and a faster degradation of galactolipids and phospholipids with a concomitant increase in sterol components (Wismer et al., 1998; Sevillano et al., 2009), are common responses to cold stress. Membranes of chilling-tolerant tissues are able to maintain the liquid-crystalline state at lower temperatures in contrast to those of chilling-sensitive, then avoiding membrane rigidity induced by cold (Marangoni et al., 1996). The secondary response to cold have been associated with oxidative stress, mediated by the generation of reactive oxygen species (ROS) such as singlet oxygen or hydrogen peroxide and by free radicals like hydroxyl and peroxyl radicals as well as superoxide anions (Prasad et al., 1994; Prasad, 1996; Sevillano et al., 2009; Hossain et al., 2012). Oxidative stress has been previously associated with chilling injury development in different cold sensitive species, as tomato (Malacrida et al., 2006), cucumber (Chen and Yang, 2013), mango fruit (Chongchatuporn et al., 2013) and also citrus fruit (Sala, 1998; Sala y Lafuente, 1999; Lafuente et al., 2004). Hence, evidences demonstrate that the generation of ROS is directly related to the inception and development of chilling injury (Purvis et al.,1995; Hodges et al., 2004).

Plant cells have developed a complex antioxidant enzymatic and non-enzymatic systems to cope with the generation of ROS during exposure to cold stress (Apel and Kirk, 2004; Foyer and Noctor, 2005). Thus, an enhancement in the activity of antioxidant enzymes has been observed in fruit stored at low temperature (Sala, 1998; Chongchatuporn et al., 2013) and the damages induced under such conditions are thought to be due to the loss of an efficient ability to scavenge cold-induced ROS (Sala, 1998; Sala and Lafuente, 1999; Sevillano et al., 2009). The major scavenger proteins found in plant cells are superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) (Hossain et al., 2012). SOD constitutes the first level of defense against superoxide radicals and catalyzes the dismutation of O_2^- to H_2O_2, another ROS that is mainly removed by the action of APX and CAT. APX dismutes H_2O_2 using AsA as an electron donor while GSH also exerts the same function for the conversion of dehydroascrobic (DHA) back into AsA, with the subsequent formation of the oxidized glutathione disulfide (GSSG) (Foyer and Noctor, 2005). GR is a rate-limiting enzyme from the ascorbate-glutathione cycle that maintains the GSH/GSSG ratio favorable for ascorbate reduction (Foyer et al., 2005).

In citrus fruit, a higher CAT activity has been associated with the natural and induced resistance to chilling during cold storage (Sala, 1998; Sala and Lafuente, 2000; Lafuente et al., 2004; Ghasemnezhad et al., 2008). Moreover, the higher ability of mature lemon fruit to cope with

CI respect to green fruit has been related to a higher activity of the enzymes CAT and SOD (Safizadej et al., 2007) since calcium and hot water dip treatments, which reduced the susceptibility of lemon fruit to CI, also provoked an increment of CAT and SOD activities (Safizadej et al., 2007). Similarly, heat-conditioning in Marsh grapefruit induced the expression of a *CAT* gene as well as reduced the incidence of CI (Maul et al., 2008; 2011). Interestingly, the protection of CI induced by heat in Fortune mandarins was notably reduced in presence of a CAT activity inhibitor (Sala and Lafuente, 2000). These results indicate the relevance of this enzyme in the heat-induced resistance to chilling. However, the role of other antioxidant enzymes in the response of grapefruit to cold or to heat-conditioning treatments is still controversial, since no consistent relationships between accumulation of *SOD* and *APX* transcripts and heat-induced chilling tolerance have been observed in Star Ruby grapefruit (Sapitnitskaya et al., 2006; Maul et al., 2008; 2011). Besides these enzymes, low molecular weight antioxidants such as AsA and GSH play also key roles in the detoxification of ROS. Apart from being a substrate of APX, AsA can also react directly with superoxide, singlet oxygen, hydroxyl radical and H_2O_2. Similarly, GSH displays the ability to directly scavenge singlet oxygen, superoxide and hydroxyl radicals (Foyer and Noctor, 2005).

We have recently reported that CI symptoms in Star Ruby grapefruit were substantially reduced or almost absent in red-colored zones of the peel. Moreover, we provided evidences showing that increasing lycopene concentration in the peel of grapefruit, by chemical or environmental manipulation, induced tolerance to CI during storage at low temperatures (Lado et al., 2015a). Carotenoids are isoprenoid-derived pigments responsible for the coloration of citrus fruit that also exhibit an important antioxidant capacity. Carotenoids are able to scavenge ROS generated during stressful conditions, acting mainly as singlet oxygen quenchers and then, carotenoid content in plant tissues may impact the antioxidant system homeostasis and the potential tolerance to the ROS-generated damage (Di Mascio et al., 1989; 1992). Then, the rational of this work was that the tolerance to CI in red, lycopene-accumulating rind of grapefruit may be due to an enhancement of the enzymatic and/or non-enzymatic antioxidant systems. To evaluate this hypothesis, total antioxidant capacity, activity and gene expression of the antioxidant enzymes GR, APX, CAT and SOD, and the antioxidant metabolites GSH and AsA were analysed in the peel of Star Ruby grapefruits with contrasting tolerance to CI. We took advantage of our previous observation that SR grapefruit growing in the field under standard environmental conditions (non-covered) developed a light-yellow coloration and were sensitive to CI upon subsequent cold storage, in contrast to bagged fruit (covered) that were red-colored with high lycopene content and more resistant to CI (Lado et al., 2015a; 2015b).

MATERIALS AND METHODS

Plant material and storage conditions

Star Ruby grapefruit (*Citrus paradisi* Macf.) was covered (C) in July at immature green stage with black plastic bags, leaving the end-bottom open to allow gas exchange (Lado et al., 2015b). The experiment was carried out in a commercial orchard located in Lliria, Valencia, Spain (39º37’20’’N, 0º33’38’’W). Three replicates of twenty fruit each for NC and C fruit were used and harvested at full maturity in December. C and NC fruit were stored at 2 ºC for 58 days plus 7 days

at 20 ºC to simulate shelf life conditions. At each sampling date, peel tissue was excised, frozen in liquid nitrogen, ground to a fine powder and stored at -80 ºC until analysis.

Chilling injury evaluation

CI symptoms in SR grapefruit appear as brown shrunken areas that increase in size and number as cold storage progressed. Three replicates of ten fruit each were inspected and scored on a scale from 0 (no injury) to 3 (severe injury) according to necrotic surface area and browning intensity. Results were expressed as CI index, which was calculated by adding the product of the number of fruit in each category multiplied by the score of each category and afterwards dividing this amount by the total number of fruit evaluated (Schirra, 1992). Results correspond to the mean ± S.E.

Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) determination

MDA concentration was measured following the procedure of Hodges et al. (1999). Frozen peel tissue (0.2 g) was homogenized in 80% cold ethanol using a tissue homogenizer (Ultra-Turrax, IKA-Werke, Staufen, Germany). Homogenate was centrifuged to pellet debris and different aliquots of the supernatant were mixed either with 20% trichloroacetic acid or with a mixture of 20% trichloroacetic acid and 0.5% thiobarbituric acid. Both mixtures were allowed to react in a water bath at 90 ºC for 1 h. After that, samples were cooled in an ice bath and centrifuged. Absorbance of the supernatant was read at 440, 534 and 600 nm against a blank. Calculation of concentration was performed as follows:

1. $[(Abs\ 532_{+TBA}) – (Abs\ 600_{+TBA}) – (Abs\ 532_{-TBA} – Abs\ 600_{-TBA})]=A$
2. $[(Abs\ 440_{+TBA}-Abs600_{+TBA}) \times 0.0571]=B$
3. MDA equivalents (nmol/mL)= $(A-B/157{,}000) \times 10^6$

Hydrogen peroxide content was estimated according to Brennan and Frenkel (1977) with slight modifications. Half gram of frozen flavedo tissue was macerated in 10 mL cold acetone and the homogenate was filtered through Whatman No.1 filter paper. Two milliliters of this filtrate were treated with 1 mL of titanium reagent (20% titanium tetrachloride in concentrated HCl, v/v, Panreac, Barcelona, Spain) and 1 mL of concentrated ammonia solution to precipitate the titanium-hydroperoxide complex. After centrifugation (at 5000 *g* for 30 min) the precipitate was dissolved in 2N H_2SO_4 and the absorbance was read at 415 nm. H_2O_2 content was calculated from a standard curve prepared by using different concentrations of H_2O_2 solutions (110-3520 nmol/mL working solutions prepared from an 888 mM stock solution).

Total antioxidant capacity measurements

Three different methods were assayed for estimating total antioxidant capacity in the peel of grapefruit: 2-2 diphenyl-1-picrylhydrazil (DPPH), 2,2'-azobis (3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) and oxygen radical absorbance capacity (ORAC).

For DPPH and ABTS assays, 0.15 g of frozen tissue was extracted with methanol 80% using a pre-chilled mortar and a pestle on an ice bath. The homogenate was filtered through two

layers of muslin cloth and centrifuged 15 min at 5000 *g* at 4 ºC. A 10 µL sample extract was used for DPPH and ABTS assays.

For DPPH determinations, a 100 µM DPPH methanolic solution was prepared and 1 mL was mixed with 10 µL of the extracted sample and allowed to stand for 30 min at 27 ºC and complete darkness. Different concentrations of ascorbic acid were used as standards as described in Girennavar et al. (2007). Absorbance was measured at 515 nm and compared to the absorbance obtained for the control (without samples or standards). Radical scavenging capacity was expressed as the inhibition percentage and was calculated using the following formula, % radical scavenging activity = (control optical density – sample optical density/control optical density) x 100 as described in Girennavar et al. (2007).

Determination of antioxidant capacity by the ABTS method was carried out as described in Girennavar et al. (2007) with slight modifications. ABTS 7 mM solution was prepared 16 h before and stored in the dark and adjusted with methanol to an absorbance of 0.7 at 734 nm at the beginning of the assay. A sample aliquot of 10 µL was added to 1 mL ABTS solution and kinetic started immediately. Measurements at 734 nm were registered each minute until five minutes. The radical scavenging capacity was calculated as described above for DPPH assay.

Determination ORAC was done according to Gillespie et al. (2007) with minor modifications. Briefly, 0.2 g of frozen peel tissue was extracted in 50% acetone using a pre-chilled mortar and a pestle on an ice bath. The homogenate was filtered through two layers of muslin cloth and centrifuged 15 min at 5000 *g* at 4 ºC. Supernatant was diluted 500 times in phosphate buffer (pH 7.0) to be used in the reaction. The reaction was done in a 96 well black microplate (Costar, Thermo-Fisher Scientific, Madrid, Spain) for fluorescence measurements. Each well contained 150 µL of 0.08 µM fluorescein (Sigma-Aldrich, Barcelona, Spain), 25 µL of diluted sample or phosphate buffer for blanks. The microplate was incubated at 37 ºC for 5 min and afterwards 25 µL of APPH (150 mM) were injected, and the kinetic measurements were initiated immediately. For the standard curve, Trolox (Sigma-Aldrich, Barcelona, Spain) concentrations from 6.25 to 50 µM were added instead of sample and the absorbance was registered. Fluorescence kinetic measurements were made in a Polarstar (BMG LABTECH, Offenburg, Germany). The excitation wavelength was set at 485 nm and the emission wavelength at 530 nm. The duration of the kinetic was 1 h at 37 ºC with one measurement per minute. The net area under curve (AUC) was calculated according to the procedure described in Gillespie et al. (2007) and compared with the blank AUC and Trolox curve to obtain Trolox equivalents/g FW.

Determination of singlet oxygen absorption capacity (SOAC)

The procedure used for SOAC determination was described in Ouchi et al. (2010) and Aizawa et al. (2011) and was followed with slight modifications. Briefly, 0.5 g of frozen peel tissue was extracted in 6 mL of cooled ethanol/chloroform/water (50:50:1, v/v/v) using a pre-chilled mortar and pestle on an ice bath and using sea sand as an abrasive. The homogenate was filtered through one layer of muslin cloth. Afterwards, 200 µL aliquot of the above-mentioned filtrate was mixed with 2 mL of 0.10 mM 2,5-diphenyl-3,4-benzofuran (DPBF, Sigma-Aldrich, Barcelona, Spain) and 1 mL of 1.5 mM endoperoxide (EP, Invitrotech, Japan). DPBF was used as an indicator of the singlet oxygen quenching capacity and EP as the singlet oxygen generator. Absorbance

changes of DPBF at 413 nm were monitored during 1 h reaction at 35 ºC. α-Tocopherol (Sigma-Aldrich, Barecelona, Spain) was used as standard and the relative SOAC value for each sample was calculated as: $(t_{1/2\ sample} - t_{1/2blank})/(t_{1/2\ \alpha Toc} - t_{1/2blank\ \alpha Toc})$* ([α-Toc g/L]/[sample g/L]).

Vitamin C determination

Ascorbic acid (AsA) and dihidroascorbic acid (DHA) were determined as described in Alós et al. (2014). Briefly, peel tissue (0.5 g) was homogenized for 1 min using a homogenizer (Polytron, Eschbach, Deutschland) with 2% metaphosphoric acid (4 mL) at medium/maximum speed. The homogenate was centrifuged for 10 min at 5000 *g* at 4 ºC. The supernatant was filtered through a C_{18} cartridge (SepPak, Waters, Spain), previously activated with 4 mL of methanol, 4 mL of water and 4 mL of 2% metaphosphoric acid. The extract was subsequently filtered through a 0.45 µm nylon filter (25 mm diameter, Análisis Vínicos, Spain). The filtrate was injected in the HPLC for AsA determination and chromatographic conditions were previously described in Alós et al. (2014).

Glutathione determination

The DTNB-GSSG reductase recycling procedure was used for determination of both total (GSH+GSSG) and GSSG levels. A 0.5 g of frozen peel tissue was homogenized in 5 mL of 5% (w/v) cold metaphosphoric acid. Homogenate was then centrifuged at 4 ºC for 30 min to pellet debris and supernatant neutralized with 0.5 M PBS (pH 7.5). Total GSH (GSH) was measured in 1 mL reaction mixture containing 125 mM PBS and 6.3 mM EDTA, 6 nM DTNB, 0.3 mM NADPH and the sample. Reaction was initiated by adding 1 unit of pure GR (Sigma-Aldrich, Barecelona, Spain) to the reaction mixture and monitored by measuring absorbance changes at 412 nm. For measuring GSSG, plant extracts were incubated at 25 ºC with 2-vinylpyridine (Fluka, Madrid, Spain) for 1 h prior to assay. The assay for GSSG was conducted as described above for total GSH. GSH and GSSG contents were estimated by interpolating slope values in a calibration curve performed with pure standards. GSH content was calculated by subtracting GSSG content from total GSH content.

Antioxidant enzymes activity determination

Protein extraction was performed using a pre-chilled mortar and a pestle on an ice bath. Briefly, 0.5 g of frozen peel tissues was extracted in 2.5 mL of extraction buffer: 100 mM potassium phosphate buffer, pH 7.5, containing 0.1% (v/v) Triton X-100 and 1% (w/v) polyvivylpyrollidone (PVP) using sea sand as an abrasive. After extraction, the mortar was rinsed with another 2.5 mL of buffer that were also collected. The homogenate was filtered through two layers of muslin cloth. The different buffers used for enzyme measurements were the following: for APX (EC1.11.1.11), 50 mM PBS (pH 7.1) supplemented with 1 mM sodium ascorbate, 0.1 mM EDTA and two drops of Triton X-100 (Panreac, Barcelona, Spain); for SOD (EC1.15.1.1), 50 mM PBS (pH 7.8) with 1.33 mM diethyl-diamino-pentaacetic acid was used; CAT (EC1.11.1.6) and GR (EC1.6.4.2) were performed in 50 mM PBS (pH 6.8) and (pH 7.5), respectively. The APX activity was determined following the depletion in absorbance at 290 nm due to AsA consumption. The SOD activity was determined following the $O_2^{\bullet -}$ induced reduction of nitroblue tetrazolium using

the xanthine-xanthine oxidase system. CAT was assayed using the hydrogen peroxide-dependent reduction of titanium chloride. The GR activity was analysed following (for 2 min) the increase in absorbance at 412 nm due to the formation of the adduct DTNB-GSH after GSSG reduction. All assays were performed at 25 ºC. Soluble protein content was determined according to (Bradford, 1976) with BSA as a standard. Enzyme activity was expressed as arbitrary U/mg protein. Further details on enzyme assays are provided in Arbona et al. (2003).

Quantitative Real time-PCR

Total RNA was isolated from the flavedo of fruit sampled at harvest and after 14, 28 and 58 days at 2 ºC and after 58 days at 2 ºC + 7 days at 20 ºC, using the RNeasy Plant Mini Kit (Qiagen, Spain) and subsequently treated with DNase (DNA free, DNase treatment & removal, Ambion). The transcripts present in 2 µg of total RNA were reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen) in a total volume of 20 µL. One µL of a 5-times diluted first-strand cDNA, containing approximately 100 ng of cDNA, was used for each amplification reaction. Quantitative real-time PCR was performed on a LightCycler 480 instrument (Roche), using the LightCycler 480 SYBRGreen I Master kit (Roche). Reaction mix and conditions followed the manufacturer's instructions. The primers used for genes are described in Appendix 1. The protocol for all genes analysed consisted of 10 min at 95 °C for pre-incubation, then 40 cycles of 10 s at 95 °C for denaturation, 10 s at 59 °C for annealing and 10 s at 72 °C for extension. Fluorescent intensity data were acquired during the extension time. Specificity of the PCR reaction was assessed by the presence of a single peak in the dissociation curve performed after the amplification steps. For expression measurements, we used the LightCycler 480 Software release 1.5.0, version 1.5.0.39 (Roche) and calculated expression levels relative to values of a reference sample using the Relative Expression Software Tool (REST, Pfaffl et al., 2002). Normalization was performed using the expression levels of the *ACTIN* gene based on previous results (Alós;Rodrigo;et al., 2014). The reference sample for expression was the value obtained for each gene on the flavedo from not covered fruit at harvest (time 0), which was set at 1. Results are expressed as the average of four independent replicates ± S.E.

Experimental design and statistical analysis

The experimental design was completely randomized. Results were expressed as mean ± standard errors (SE) and for continuous variables with normal distribution, data were subjected to analysis of variance and differences between means determined by T unpaired test, setting significance level at $p<0.05$. In the case of gene expression analysis, data were analysed using the software REST 2009 (http://genequantification.de/rest-2009.html) to determine statistical significance. A pair wise fixed reallocation randomization test was applied, which makes no assumptions about the distribution of the observations.

RESULTS

CI Incidence in SR fruit

To evaluate the involvement of the different components of the antioxidant system in the tolerance of the red peel of grapefruit to CI, we used fruit of the Star Ruby variety that were bagged (C) or left un-bagged (NC) on the field at immature green stage and at the harvest time (December) developed an homogeneously red or the typical yellow colouration, respectively (Lado et al., 2015b). Analysis of carotenoid content revealed a 4-fold increment in total carotenoids and a lycopene concentration around 75 times higher in C than in NC fruit (Lado et al., 2015a). Storage at 2 ºC produced a rapid and more intense development of CI symptoms in NC than in C grapefruit. After 28 days storage, the incidence of chilling was near 6-times higher in C fruit as well as was the severity of the damage. At the end of the storage and simulation of shelf-life, all NC fruit were affected by CI whereas only 40% of those covered developed chilling damage and of low severity (2.2 vs 0.8) (Table 1). These results confirmed the tolerance of red-coloured grapefruit to CI.

Table 1. Chilling injury index (CI) and incidence of affected fruit (%) in non-covered (NC) and covered (C) Star Ruby grapefruit stored for up to 58 days 2 ºC followed by additional 7 days at 20 ºC. *Letters indicate significant differences between NC and C fruit for each sample date ($p<0.05$, T unpaired test). The absence of letters indicates no significant differences.

	CI Index (0-3)		Incidence (%)	
Days at 2ºC	NC	C	NC	C
0	0	0	0	0
14	0.1 a	0 b	6.7 a	0 b
28	0.8 a	0.07 b	73 a	13 b
42	1.6 a	0.4 b	87 a	27 b
58	1.9 a	0.5 b	93 a	33 b
58+7 (20ºC)	2.2 a	0.8 b	100 a	40 b

Evaluation of oxidative stress damage: hydrogen peroxide and lipid peroxidation during cold storage

Since CI damage in citrus fruit has been associated to ROS production and to membrane lipids peroxidation, the involvement of oxidative stress in chilling-sensitive and tolerant SR fruit was evaluated by the analysis of hydrogen peroxide production and lipid peroxidation (MDA content) (Fig. 1). Interestingly, hydrogen peroxide at harvest was about a 37% lower in the peel of covered fruit, indicating lower oxidative stress in these fruits. After cold storage, H_2O_2 concentration increased more rapidly in C than in NC fruit, to be almost similar in C and NC fruits from week 2 to the end of the storage period. Simulation of shelf-life at 20 ºC increased H_2O_2 in non-covered but not in C fruit (Fig. 1A). MDA concentration was also higher in NC at harvest while in C fruit experienced a marked increment to reach similar values to NC fruit at 7 and 14 days of storage. Differences between sensitive and tolerant samples were detectable again after 28 days at 2 ºC, when MDA concentration was around 30% higher in the peel of CI-damaged than in CI-tolerant C fruit (Fig. 1B).

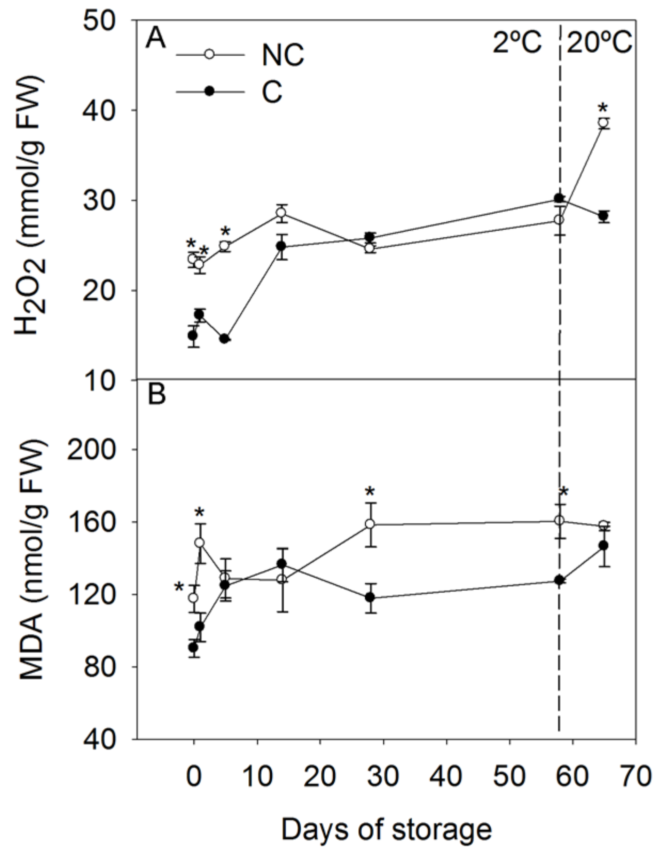

Figure 1. Hydrogen peroxide-H2O2 (A) and malondialdehyde (MDA) (B) in the peel of non-covered (NC) and covered (C) Star Ruby grapefruit stored for up to 58 days 2 ºC followed by additional 7 days at 20 ºC. *Asterisks indicate significant differences between NC and C fruit for each sample date ($p<0.05$, T unpaired test).

Total antioxidant capacity in CI-sensitive and CI-tolerant fruit

Oxidative stress triggers different mechanisms to protect plant cells from ROS damage. In order to evaluate the possible relationship between total antioxidant capacity and the tolerance of the peel of C grapefruit to CI, three methods were used to quantify the ability to avoid oxidative damage: DPPH, ABTS and ORAC. DPPH-antioxidant capacity was higher in control NC compared to C fruit at harvest and during storage, and increased in the peel of both fruit with the storage time. A similar behaviour was observed for NC and C fruits with the ABTS assay, and differences between samples were maintained during cold stored and lost after simulation of shelf-life (Table 1). ORAC assay did not reveal differences between NC and C at harvest, but after 2 weeks of cold storage, the values were higher in NC than C fruit (Table 1). Collectively, analysis of antioxidant activity by these 3 methods showed a higher capacity in CI-sensitive fruit than in CI-tolerant fruit which increased with the time of cold storage.

Table 2. Comparison of total antioxidant capacity in the peel of non-covered (NC) and covered (C) Star Ruby grapefruit stored for up to 58 days 2 ºC followed by additional 7 days at 20 ºC. Total antioxidant activity was determined by three different methods: DPPH (% of reduction), ABTS (% of reduction) and ORAC (μmol/TEg FW). *Letters indicate significant differences between NC and C fruit for each sample date (p<0.05, T unpaired test). The absence of letters indicates no significant differences.

	DPPH (% reduction)		**ABTS (% reduction)**		**ORAC (μmol/TEg FW)**	
Days at 2ºC	NC	C	NC	C	NC	C
0	29.0±2.8 a	18.9±2.8 b	40.7±2.6 a	29.5±2.9 b	45.7±3.9	43.1±4.6
1	31.3±1.0 a	23.8±2.4 b	30.5±0.8 a	17.8±1.2 b	37.6±3.1	42.2±3.3
5	32.4±2.6 a	26.9±3.9 b	47.6±0.2 a	25.0±0.6 b	31.9±3.6	35.3±1.3
14	28.6±1.7 a	22.8±0.4 b	50.5±4.5 a	34.1±0.8 b	44.6±1.4 a	33.5±1.3 b
28	36.5±4.9 a	19.6±2.4 b	67.9±6.2 a	40.2±0.9 b	39.9±1.9 a	32.9±1.3 b
58	37.5±1.1 a	25.8±2.7 b	49.8±2.2 a	37.9±1.7 b	58.6±3.0 a	48.2±3.1 b
58+7 (20ºC)	34.1±1.2 a	25.5±1.1 b	41.7±1.10	42.2±0.9	59.6±2.5 a	49.9±1.7 b

Singlet oxygen absorption capacity (SOAC) in CI sensitive and CI-tolerant fruit

Based on the differences on carotenoid composition between NC and C fruit which were thoroughly described in a previous manuscript (CI-resistant grapefruit contained higher carotenoid and lycopene concentration than NC, CI-sensitive fruit; Lado et al., 2015a) and considering the role of carotenoids as efficient singlet oxygen quenchers (Krinsky, 1989; Di Mascio et al., 1989), the ability of peel extracts to scavenge singlet oxygen reactive species (SOAC) was measured. Singlet oxygen quenching capacity was around 70% lower in the peel of yellow CI-sensitive fruit than in lycopene-accumulating C fruit (Fig. 2). After 30 days of cold storage, in both fruit SOAC capacity decreased to slightly increase latter on, but it is noticeable that remained always higher (3-3.5 times) in C than in NC fruit (Fig. 2).

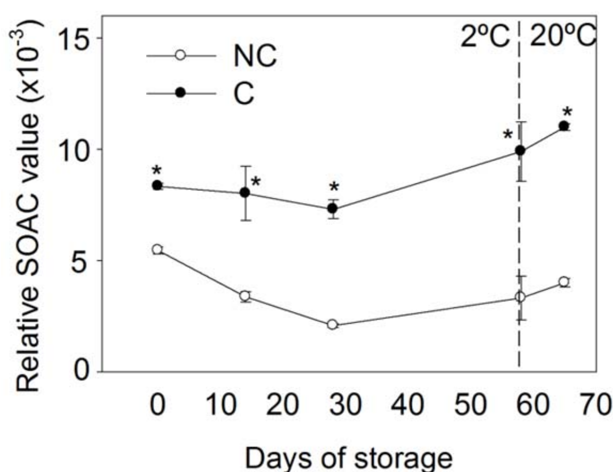

Figure 2. Singlet oxygen absorption capacity (SOAC), expressed as relative values, in the peel of non-covered (NC) and covered (C) Star Ruby grapefruit stored for up to 58 days 2 ºC followed by additional 7 days at 20 ºC. *Asterisks indicate differences between NC and C fruit for each sample date (p<0.05, T unpaired test).

Non-enzymatic antioxidant system: glutathione (GSH) and ascorbic acid (AsA) in CI-sensitive and CI-tolerant fruit

To explore the potential contribution of other non-enzymatic antioxidants in the differential sensitivity to CI between NC and C fruit, the concentration of two powerful antioxidant molecules, GSH (as the sum of GSH+GSSG) and AsA was also determined. The response of these metabolites to cold storage was different. With the exception of fruit stored 58 days at 2 ºC, total GSH was similar between C and NC grapefruit and significantly declined after fruit transference to shelf-life conditions (Table 3). By contrast, AsA content was 2.5-times higher in the peel of NC than C fruit at the harvest time. Cold storage considerably reduced AsA, about a 31% in NC fruit and 18% in C fruit, to increase again upon fruit rewarming (Table 3). In general, AsA concentration was always higher in the peel of CI-sensitive grapefruit whereas no differences were detected for GSH.

Table 3. Glutathione (nmol/g FW) and ascorbic acid (AsA) content (mg/100gFW) in the peel of non-covered (NC) and covered (C) Star Ruby grapefruit stored for up to 58 days 2 ºC followed by additional 7 days at 20 ºC. *Letters indicate significant differences between NC and C fruit for each sample date ($p<0.05$, T unpaired test). The absence of letters indicates no significant differences.

	Glutathione (nmol/g FW)		**AsA (mg/100g FW)**	
Days at 2ºC	NC	C	NC	C
0	109±8	125±8	307±11 a	122±3 b
1	92,6±3	93±9	289±12 a	127±3 b
5	119±5	120±3	200±4 a	112±2 b
14	125±3	110±6	217±6 a	115±1 b
28	147±1	156±2	226±4 a	108±9 b
58	114±7b	141±3a	211±3 a	101±5 b
58+7d at 20ºC	88±9	96±4	250±1 a	120±3 b

Enzymatic antioxidant system: GR, APX, CAT and SOD enzyme activities and genes expression in CI-sensitive and CI-tolerant fruit

Enzymatic activity and transcriptional analysis of key enzymes involved in the detoxification of ROS in plant cells: GR, APX, CAT and SOD, were determined in the peel of CI-sensitive and CI-tolerant grapefruit. GR and APX experienced minor changes in enzymatic activity during cold storage and increased slightly after simulation of shelf-life, but with no significant differences between NC and C fruit (Fig. 3). Two isoforms of GR (*GR1 and GR2*) gene were analysed, and results shown a similar pattern of mRNAs accumulation in NC and C fruit, with an increment during cold storage to sharply decline after shelf-life (Fig. 3A). On the other hand, expression of the *APX2* and *APX4* isoforms were also similar between both fruit and did not show important changes either during cold or shelf-life (Fig. 3B).

CAT activity was slightly higher in the peel of C fruit than in NC and in both fruit remained nearly constant during cold storage. After removing fruit from cold storage, CAT activity experienced a sharp increment, reaching values (14-16 U/mg protein), around 4-times higher than during cold storage (Fig. 4A). Accumulation of transcripts corresponding to *CAT1* and *CAT2*

isoforms increased progressively during cold storage and markedly declined after transference to 20 ºC, being virtually similar in the peel of both C and NC fruit (Fig. 4A). SOD activity showed a different behaviour increasing during the first 14 days of cold storage in the peel of CI-tolerant fruit, and no differences between both types of fruit were detected latter on. In contrast to CAT, SOD enzyme activity decreased after fruit transference to 20 ºC, but again without differences between C and NC fruit. Accumulation of the transcripts corresponding to the *MnSOD*, *CuZnSOD* and *FeSOD* genes were determined (Fig. 4B). *MnSOD* and *CuZnSOD* mRNAs accumulated progressively during cold storage in both NC and C fruit but minor changes were observed in *FeSOD* transcripts. Similarly to *CAT*, expression of *MnSOD* and *CuZnSOD* genes declined upon stimulation of shelf-life at 20 ºC, whereas only a small increment was observed for the *FeSOD* isoform (Fig. 4B).

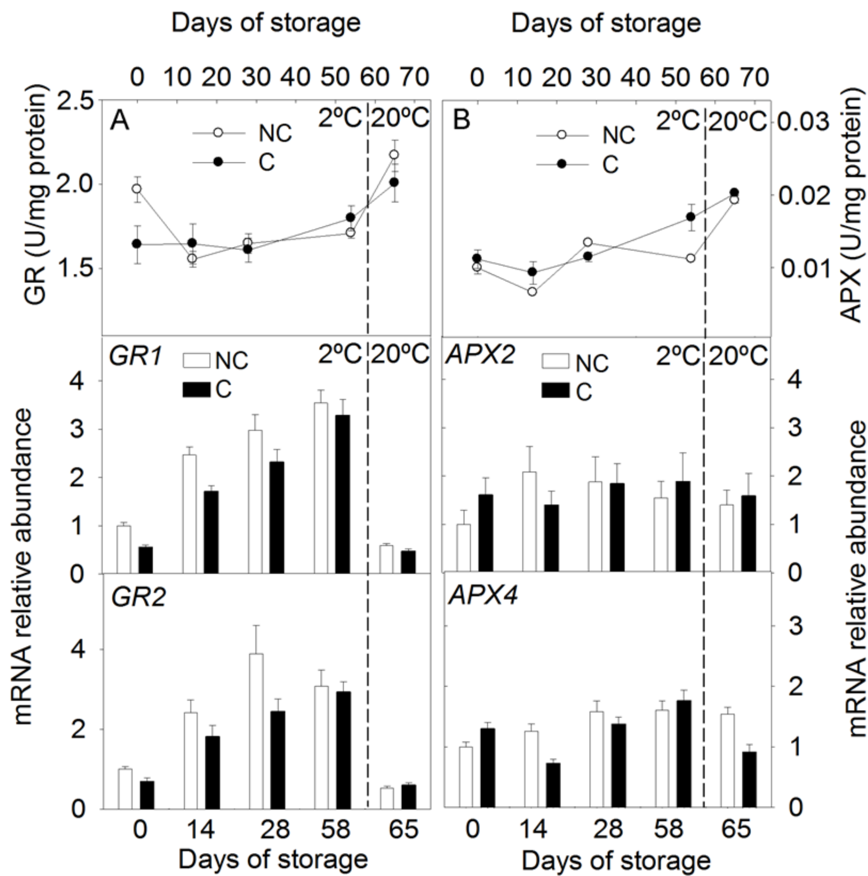

Figure 3. Glutathione reductase activity and relative expression of *GR1* and *GR2* isoforms (A) and ascorbate peroxidase activity and relative expression of *APX1* and *APX2* isoforms (B) in the peel of non-covered (NC) and covered (C) Star Ruby grapefruit stored for up to 58 days 2 ºC followed by additional 7 days at 20 ºC. * Asterisks indicate differences between NC and C fruit for each sample date ($p<0.05$, T unpaired test for enzymatic activity and pair wise fixed reallocation randomization test for gene expression). The absence of asterisks indicates no significant differences.

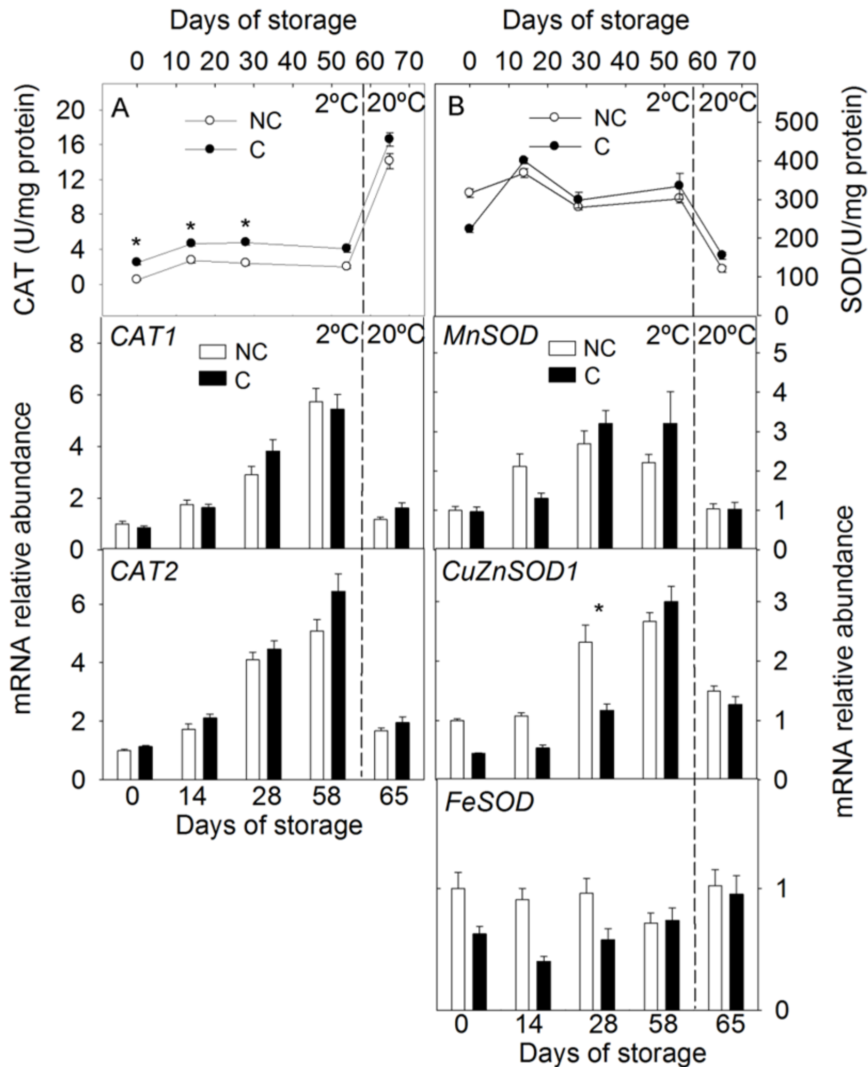

Figure 4. Catalase activity and relative expression of *CAT1* and *CAT2* isoforms (A) and superoxide dismutase activity and relative expression of *MnSOD*, *CuZnSOD* and *FeSOD* genes (B) in the peel of non-covered (NC) and covered (C) Star Ruby grapefruit stored for up to 58 days 2 ºC followed by additional 7 days at 20 ºC. *Asterisks indicate differences between NC and C fruit for each sample date ($p<0.05$, T unpaired test for enzymatic activity and pair wise fixed reallocation randomization test for gene expression). The absence of asterisks indicates no significant differences.

DISCUSSION

In a previous study we demonstrated that the red zones of the peel of grapefruit were tolerant to CI during storage at low temperature (Lado et al., 2015a). Increasing lycopene content, by chemical and environmental manipulation, in the rind of both the white Marsh and the red Star Ruby grapefruit induced tolerance to CI, suggesting that high levels of the red carotenoid lycopene may be responsible or directly involved in the induction of tolerance to CI. In citrus fruit, chilling damage has been associated to an oxidative burst process (Sala, 1998) and the inability of the cells to cope with the cold-induced ROS determines an increment in membrane lipid peroxidation that

could eventually trigger cell death (Sevillano et al., 2009; Hossain et al., 2012). Moreover, tolerance to cold induced by various heat conditioning treatments in fruit of different citrus species and varieties has been also related to an activation of the antioxidant enzymatic systems (Sala and Lafuente, 1999, 2000; Lafuente et al., 2004; Sapitnitskaya et al., 2006; Maul et al., 2008, 2011). Since lycopene is recognized as a carotenoid with potent antioxidant properties (Krinsky, 1989), our hypothesis established that the tolerance to CI in the red rind of grapefruit could be due to alterations of the enzymatic or non-enzymatic antioxidant systems.

The incidence of CI in NC grapefruit that were exposed in the field to standard environmental conditions, increased substantially during the first month of cold storage, and 73% of the fruit showed CI symptoms, as compared with only 13% in covered fruit (Table 1). During the second month of cold-storage, CI symptoms increased in severity, reaching a value near 2 (medium damage) in NC, whereas in those with high lycopene content, CI index was always lower than 1, in agreement with previous results (Lado et al., 2015a). Analysis of H_2O_2 and MDA contents in the peel of SR grapefruit revealed a higher content in NC than in C at the harvest time (Fig. 1A, B). Lipid peroxidation, evaluated through MDA content, is one of the most widely used indicators of membrane damage due to the existence of free radicals that react with fatty acids and phospholipids. Our results indicate that fruit bagging also affected the incidence of oxidative stress under field conditions, since flavedo cells of NC fruit appears to be more exposed to oxidative stress, as indicated by a higher H_2O_2 content and a more intense membrane damage, than those of C fruit, which may be also related to the different lycopene concentration. However, H_2O_2 content increased rapidly in C fruit with the exposure of fruit to cold storage, reaching values similar to that of NC after 3 weeks (Fig. 1A). These observations indicate that the increase in H_2O_2 is a response of the flavedo cells to cold stress and not necessarily related to the development of CI symptoms, since during the second month of storage (when the differences in chilling damage between both type of fruit were noticeable), the content of H_2O_2 was the same (Table 1 and Fig. 1A). However, the concentration of MDA after two weeks of storage, when CI symptoms were initiated, was lower in CI-resistant (C) than in CI-sensitive (NC) fruit (Fig. 1B). Thus, the changes in MDA appear to be an early response to cold stress occurring in both chilling tolerant and sensitive fruit, and also linked to the development of chilling damage after prolonged cold storage. ROS production and MDA content have been associated to CI incidence in citrus fruit (Santini et al., 2013; Siboza and Bertling, 2013), as well as in other cold sensitive fruit such as tomato (Zhang et al., 2013).

In order to understand if the flavedo cells of CI-tolerant and sensitive grapefruit have differential ability to restore the redox equilibrium, total antioxidant capacity was determined by three different methods: ABTS, DPPH and ORAC (Table 2). The first two are based on electron transfer and estimate the ability of a potential antioxidant to transfer one electron to reduce any compound, whereas the ORAC method measures the ability of an antioxidant to quench a mix of free radicals (mainly peroxyl radicals and superoxide) (Huang et al., 2005). The three assays used revealed a higher total antioxidant capacity in the peel of NC, chilling-sensitive grapefruit. Differences were evident in freshly harvested fruit and during the whole storage period (Table 2). This higher antioxidant capacity could be also related to the higher content in AsA measured in these fruit (Table 3). These results were not unexpected and reinforced previous observation that

the peel of NC fruit is exposed to a stronger oxidative stress than that of C fruit, and should have stimulated earlier the antioxidant mechanisms to cope with the stressful field conditions. Moreover, the three assays also revealed an increment in total antioxidant capacity with the time of exposure to cold storage, but the differences between both types of fruit were maintained (Table 2). Thus, it should be suggested that the stimulation of the antioxidant capacity in grapefruit appears to be a general response induced by cold stress and that these mechanisms in NC fruit may not to be efficient enough to protect fruit from the cold-induced damage.

Since analysis of total antioxidant capacity, by conventional procedures, does not explain the tolerance to CI in lycopene-accumulating rind of covered grapefruit, and that carotenoids present the ability to quench singlet oxygen (Krinsky, 1989; Di Mascio et al., 1989), we analysed this specific feature in lipophilic peel extracts of C and NC fruit, by the SOAC method (Ouchi et al., 2010; Aizawa et al., 2011). This method has been recently proposed as a reliable system for evaluating the contribution of carotenoids to the antioxidant response in food extracts (Aizawa et al., 2011). As expected, the SOAC relative value of CI-tolerant fruit at harvest and during cold-storage was nearly 70% higher than that of chilling-sensitive fruit (Fig. 2). These results provide evidences of the involvement of singlet oxygen in the deleterious process originating CI damage in grapefruit. Due to its powerful oxidant nature, singlet oxygen causes direct damage to essential biomolecules such as proteins, nucleic acids and lipids in membranes, driving to cell death (Fischer et al., 2013). The ability of carotenoids to quench $^1O_2$ increases with the number of conjugated double bonds, and lycopene with the presence of 11 conjugated double bounds is one of the most effective quenchers (Krinsky, 1989; Di Mascio et al., 1989; 1992; Aizawa et al., 2011; Fisher et al., 2013). Then, the fact that the presence of high lycopene concentrations (Lado et al., 2015a) in peel extracts of covered, CI-tolerant fruit, originated a bigger ability to scavenge singlet oxygen, indicated a protective role of this pigment in the perception of cold stress and the development of CI in the flavedo of citrus fruit. Moreover, singlet oxygen has been also proposed to act as a signal triggering programmed cell death (Apel and Hirt, 2004; Wagner et al., 2004). It is then conceivable that singlet oxygen could be also acting as a signaling molecule for CI development in SR grapefruit, activating the genetically program of stress response that finally would conduct to cell death and therefore, to the appearance of peel damage symptoms.

Other components of the antioxidant systems were also evaluated in the differential response of C and NC grapefruit to CI. Non-enzymatic antioxidants like GSH and AsA act as substrates for the action of antioxidant enzymes as well as display the capacity to detoxify different ROS by themselves (Noctor et al., 1998). Moreover, a higher AsA content has been associated to a lower incidence of CI in other cold-sensitive fruit such as tomato (El Airaj et al., 2013), cucumber (Qian et al., 2013) and mango (Chongchatuporn et al., 2013). However, glutathione and AsA displayed opposite pattern of accumulation during cold storage of grapefruit and not consistent differences in total GSH were detected between CI-sensitive and CI-tolerant fruit. AsA content was higher in the peel of NC, CI-sensitive fruit than in C fruit and in both cases it declined during cold exposure (Table 3). Taken together, these results indicate that the changes in these two antioxidant molecules do not appear to be related to the tolerance to CI observed in Star Ruby grapefruit.

Activity and gene expression analysis of ROS detoxifying enzymes, mainly GR, APX, CAT and SOD, revealed a complex interplay in the response of grapefruit to cold and to CI.

Unexpectedly, enzymatic activity of GR, APX and SOD showed minor differences between CI-sensitive and CI-tolerant fruit and therefore, their relative contribution to the tolerance to CI seems to be marginal (Fig. 3 and 4B). A similar absence of relationship between CI incidence and the activity of these enzymes has been also described in cucumber (Qian et al., 2013; Chen et al., 2013), mango (Chongchatuporn et al., 2013) and in Fortune mandarin (Sala et al., 1999). However, a higher SOD and APX activities were associated to a lower CI incidence in tomato (Zhang et al., 2013), showing that the involvement of antioxidant enzymes in the responses to chilling damage is species-dependent. On the other hand, CAT enzyme, responsible for transforming H_2O_2 into water and oxygen, showed a slightly higher activity in C (CI-tolerant) than in NC (CI-sensitive) fruit during cold storage and could be partially contributing to the differential CI sensitivity. This higher enzyme activity during cold storage was coincident with lower hydrogen peroxide concentration in C fruit, the main substrate for the enzyme. These results are consistent with previous observation in citrus fruit in which a higher activity of this enzyme was associated with a lower incidence to CI (Sala et al., 1999; Sala et al., 2000; Lafuente et al., 2004; Ghasemnezhad et al., 2008) and suggest that CAT may also contribute to the antioxidant protection conferred by carotenoids against the cold-induced oxidative burst.

It is worth to mention that most of these antioxidant enzymes, as *CAT* and *GR* isoforms as well as for *MnSOD* and *CuZnSOD*, were transcriptionally induced in response to cold in both sensitive and tolerant fruit, being more likely an early cold-response than a protective mechanism. On the other hand, no relevant changes were registered for the expression of *FeSOD* or *APX* isoforms (*APX1* and *APX2*) in response to cold, suggesting that these specific isoforms are not temperature-responsible genes (Fig. 3B and 4B). Induction of *CAT* expression was also described in cold-stored Star Ruby fruit (Sapitnitskaya et al., 2006), reinforcing the motion that *CAT* induction is part of the defense mechanisms of the fruit to cope with cold. Moreover, our results also showed that low temperatures exert a detrimental control in GR and CAT enzymatic activities, since the stimulation in gene expression (6 and 4 times, respectively) was not immediately mirrored by a corresponding increase in enzymatic activity, being significantly activated after cold removal (Figs. 3A and 4A).

In summary, tolerance of the red, lycopene-accumulating, peel of grapefruit to chilling injury was not associated with an enhancement of neither total antioxidant capacity nor glutathione or AsA content, indicating that such antioxidant responses may be cold-mediated and not directly linked to chilling tolerance. Increasing lycopene content in the peel significantly boosted singlet oxygen scavenging capacity, determined by the SOAC assay, which appears to be the main effect of this carotene in the induction of tolerance to CI.

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SUPPLEMENTARY MATERIAL

Appendix 1

Table A1. Sequences of the primers used for quantitative real-time PCR. The genes analysed were *ascorbate peroxidase (APX1* and *APX2*), *catalase* (*CAT1* and *CAT2*), *CuZn superoxide dismutase* (*CuZn-SOD1), Fe superoxide dismutase (FeSOD), glutathione reductase (GR1 and GR2), Mn superoxide dismutase (MnSOD)* and *ACTIN*.

Primer	Orientation[a]	Gene	Primer sequence (5'-3')
MJ467	S	*GR1*	AGATCATGGTGAAGAGATTGTTGCAGA
MJ468	AS	*GR1*	ACCTTCACAGCTCCTGTTTGATCA
MJ469	S	*GR2*	ACTAATAAAGGAACAGTTGATGGCTTCTC
MJ470	AS	*GR2*	TCAACCTCTATAGCTCCATTCTTGGTC
MJ397	S	*APX1*	CGAAATGTGCGGCGTCGG
MJ398	AS	*APX1*	CCCTTCGAGGCCACTCCTC
MJ498	S	*APX2*	TGGAAGACCGGACAAATCTGATCCG
MJ499	AS	*APX2*	TTATCACTCAGACCCATGTGGCCG
MJ419	S	*CAT1*	GCCAGTGCTAAGGGTTTCTTTGAG
MJ420	AS	*CAT1*	GCTCATGGATAACAGTGGAGAAGCG
MJ421	S	*CAT2*	GCAGCTGGAAACTATCCTGAGTGG
MJ422	AS	*CAT2*	ATCCCTGTGCATGAAATTCATGAAACC
MJ407	S	*FeSOD*	ACCACTCCTTACTATTGATGTTTGGGAG
MJ408	AS	*FeSOD*	ACTACTTCCCATGACACAAGCTTGTC
MJ401	S	*CuZnSOD1*	CACGTAGCACAATGGTGAATGCAAC
MJ402	AS	*CuZnSOD1*	GGGGCTCCAGTTGTTATCCAAACATTAT
MJ399	S	*MnSOD*	CAGCTTCATCACCAGAAGCA
MJ400	AS	*MnSOD*	TCCGCCGTTGAACTTGATA
ACTINF	S	*ACTIN*	TTAACCCCAAGGCCAACAGA
ACTINR	AS	*ACTIN*	TCCCTCATAGATTGGTACAGTATGAGAC

[a] S, sense; AS, antisense

3.4. CAPÍTULO IV.

Fruit shading enhances peel colour, carotenes accumulation and chromoplast differentiation in red grapefruit

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Abstract

The distinctive colour of red grapefruits is due to lycopene, an unusual carotene in citrus. It has been observed that the red 'Star Ruby' (SR) grapefruits grown inside the tree canopy develop an intense red colouration than those exposed to higher light intensities. In order to investigate the effect of light on SR peel pigmentation, fruit were bagged or exposed to normal photoperiodic conditions and changes in carotenoids, expression of carotenoid biosynthetic genes and plastid ultrastructure in the peel were analysed. Light avoidance accelerated chlorophyll breakdown and induced carotenoid accumulation, rendering fruits with an intense colouration. Remarkably, lycopene levels in the peel of shaded fruits were 49-fold higher than in light-exposed fruit while concentrations of downstream metabolites were notably reduced, suggesting a bottleneck at the lycopene cyclization in the biosynthetic pathway. Paradoxically, this increment in carotenoids in covered fruit was not mirrored by changes in mRNA levels of carotenogenic genes, which were mostly up-regulated by light. In addition, covered fruits experienced profound changes in chromoplast differentiation and the relative expression of genes related to chromoplast development was enhanced. Ultrastructural analysis of plastids revealed an acceleration of chloro- to chromoplast transition in the peel of covered fruits concomitantly with development of lycopene crystals and plastoglobuli. In this sense, an accelerated differentiation of chromoplast may provide biosynthetic capacity and a sink for carotenoids without involving major changes in transcript levels of carotenogenic genes. Light signals seem to regulate carotenoid accumulation at the molecular and structural level by influencing both biosynthetic capacity and sink strength.

INTRODUCTION

Carotenoids are isoprenoid-derived pigments accumulated in the peel and pulp of citrus fruit and are responsible for the high diversity of colourations within this crop (Gross 1987). During the last decade considerable effort has been directed at trying to understand how colour and carotenoid composition is regulated in citrus fruits, identifying key genes of the pathway and key metabolic steps (Alquézar et al. 2008, Kato et al. 2004, Kato 2012, Rodrigo et al. 2004, 2013). Mature oranges preferably accumulate xanthophylls, mainly 9-*cis*-violaxanthin (up to 80% of total carotenoids), which are responsible for their typical pale orange-yellowish colour, while β-cryptoxanthin and the apocarotenoid β-citraurin are also present in mandarin fruits, contributing to the intense orange colouration displayed by some varieties (Rodrigo et al. 2013). On the other hand, mature white grapefruits accumulate minute amounts of carotenoids, mainly phytoene and violaxanthin, while red grapefruits are able to accumulate large amounts of linear carotenes, mainly phytoene, phytofluene and the red lycopene in both peel and pulp (Alquézar et al. 2013). Furthermore, levels of carotenoids are similar in the peel and the pulp of red grapefruits, contrary to the common feature in other citrus fruits that display much higher carotenoid content in the peel than in the pulp (Alquézar et al. 2013, Rodrigo et al. 2013).

The peel of immature citrus fruit accumulates mainly lutein and minor proportions of other chloroplastic carotenoids (zeaxanthin, β- and α-carotene). At the onset of fruit colouration, lutein declines and almost disappears in parallel to a marked increase of coloured carotenoids, mostly β,β-xanthophylls (Kato et al. 2004, Rodrigo et al. 2004). These changes are transcriptionally coordinated with a parallel up-regulation of genes of the carotenoid biosynthetic pathway (Fig. 1). The process is initiated by the up-regulation of the phytoene synthase (*PSY*) (the first committed step of the pathway) and followed by an increment in the expression of phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS*), β-carotene hydroxylase (*β-CHX*) and the induction of the chromoplast-specific lycopene cyclase 2 (*βLCY2*) genes (Alquézar et al. 2008, 2009, 2013, Kato et al. 2004, Rodrigo et al. 2004).

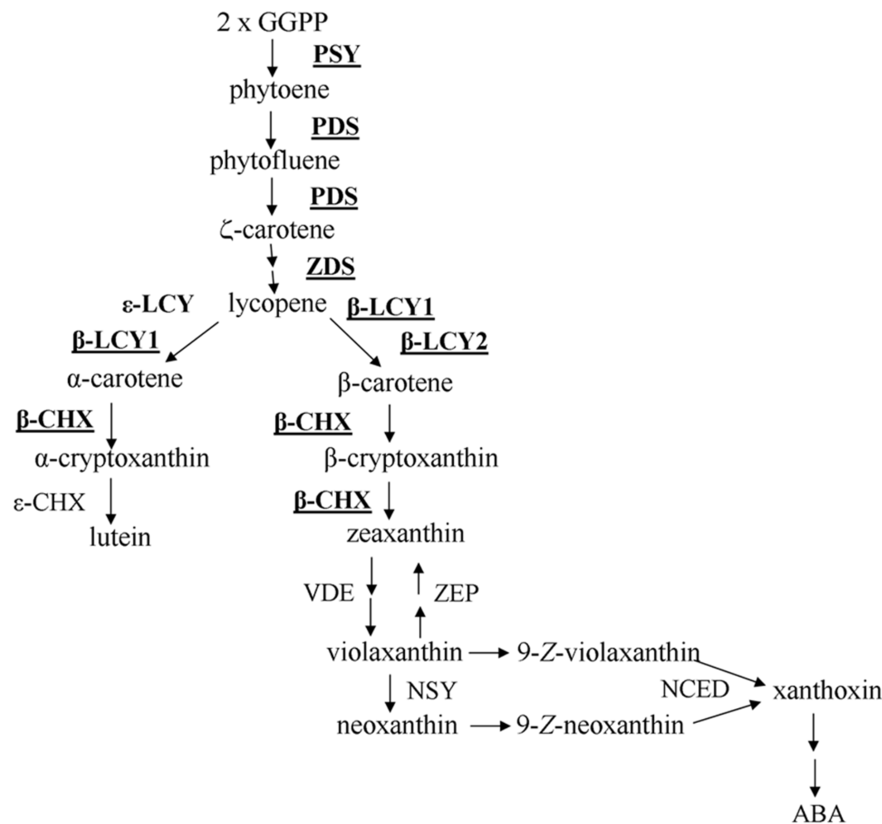

Figure 1. Schematic diagram of the biosynthesis pathway of carotenoids in citrus fruit. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ- carotene desaturase; βLCY1, lycopene β-cyclase 1; βLCY2, lycopene β-cyclase 2; βCHX, β-carotene hydroxylase; εCHX, ε-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase; NCED, 9-cis-epoxycarotenoid dioxygenase; ABA, abscisic acid. Genes underlined were analysed in this study.

Accumulation of lycopene in citrus fruit is of particular interest since it is an unusual feature restricted to only a few species such as grapefruit (*Citrus paradisi*), pummelo (*C. grandis*) and the flesh of some orange (*C. sinensis*) mutants (Alquézar et al. 2008, 2013, Liu et al. 2007). Recent studies indicate that differential mechanisms may be operating in lycopene-accumulating mutants (Alquézar et al. 2009, Costa et al. 2012, Mendes et al. 2011, Pan et al. 2009, Xu et al. 2010). In the red 'Hong Anliu' orange, a higher expression of *PSY* and *ZDS* genes and lower of *βLCYs* compared to the parental variety appears to be a key factor triggering lycopene accumulation (Xu et al. 2010). Similarly, in the red grapefruits 'Star Ruby' and 'Flame', the presence of lycopene seems to be related to a lower expression level of the fruit-specific *βLCY2* gene (Alquézar et al. 2009, 2013, Mendes et al. 2011) while in Cara-Cara orange it has been associated with an enhanced expression of the genes of the methyl-D-erythritol-4-phosphate (MEP) pathway (Alquézar et al. 2008), the main route supplying precursors for carotenoid biosynthesis. The flux into the MEP pathway is controlled by two key enzymes: the 1-deoxy-D-xylulose-5-phosphate synthase (DXS), located upstream, and the hydroxymethylbutenyl diphosphate reductase (HDR), downstream in the pathway, constituting two regulatory steps that directly influence carotenoid accumulation (Botella-Pavía et al. 2004, Peng et al. 2013). At the end of the MEP

pathway, the formation of the key precursor geranyl geranyl pyrophosphate (GGPP), which constitutes a key branch point in isoprenoid biosynthesis in plants, is catalyzed by the enzymes GGPP synthases (GGPPS), a complex family of different isoforms that vary in expression, localization and activity (Beck et al., 2013). Most GGPP synthases have been shown to be localized in plastids and are directly related to carotenoid accumulation *in vitro* (Thabet et al., 2012). Their gene transcripts levels and enzymatic activity were strongly induced during colour change in ripening pepper fruits (Kuntz et al., 1992).

Carotenoid synthesis takes place inside plastids (chloro- and chromoplasts) (Gross 1987) and the differentiation from chloroplast to chromoplasts is concomitant with massive carotenoid accumulation during fruit ripening (Cazzonelli and Pogson 2010, Gross 1987). The amount of carotenoids accumulated in plastids is determined by both the equilibrium between biosynthesis and degradation and the capacity to develop sink structures (Cazzonelli and Pogson 2010). Chromoplasts develop diverse structures for carotenoid storage, varying in shape and size, depending on the fruit and it`s developmental stage (Gross 1987, Sitte 1980). The predominant structures present inside chromoplasts are related to the type of carotenoid accumulated (Wang et al. 2013).The most common structure for carotenoid accumulation inside citrus peel chromoplasts are the spherical plastoglobuli which increase in abundance as fruit ripen (Cao et al. 2012, Gross 1987). In the peel of 'Valencia' oranges and 'Goliath' pummelo chromoplasts a combination of plastoglobuli and well organized parallel achlorphyllous membranes have been observed (Gross, 1987). On the other hand, crystals were proposed as the predominant structures for lycopene accumulation in tomato fruit (Nogueira et al. 2013, Schweiggert et al. 2011, Simkin et al. 2007) and were also present in lycopene-accumulating grapefruits (Cao et al. 2012).

Different proteins, mainly chaperones, are involved in the ultrastructural changes that take place during chromoplast differentiation. Among them are the fibrillins or plastoglobulins (FIBs or PAPs), the orange (Or) protein and small heat shock proteins (sHSPs). FIBs are the predominant proteins inside the plastoglobuli and are thought to assist in the globular-fibrillar transition which allows for the storage of high amounts of newly synthetized carotenoids during chloro- to chromoplast conversion (Simkin et al. 2007). An increased accumulation of *FIBs* (*CitPAP*) transcripts was described in citrus fruit during the colour change (Moriguchi et al. 1998). Similarly, the overexpression of *Or* gene in sweet potato led to an increase in carotenoids, mainly β-carotene and lutein (Kim et al. 2013), while overexpression of the *Or* gene from *A. thaliana* in rice callus caused a massive carotenoid accumulation (Bai et al., 2014). Other proteins involved in chromoplast differentiation are the sHSPs, which are thought to be directly related to fruit ripening (Neta-Sharir et al. 2005, Pan et al. 2012). They are abundant in orange chromoplasts (Zeng et al. 2011) and differentially expressed among citrus varieties (Pan et al. 2012).

Light, both intensity and quality, is one of the most crucial environmental factors influencing carotenoid accumulation in plant tissues (reviewed in Pizarro and Stange 2009). It has been shown that the MEP pathway and *PSY* transcription are stimulated by light (Botella-Pavía et al. 2004, Toledo-Ortiz et al. 2010). Moreover, light induced carotenoid biosynthetic genes in photosynthetic tissues (Simkin et al. 2003), in addition to enhanced carotenoid accumulation in tomato fruit (Azari et al. 2010, Schofield and Paliyath 2005). In contrast, darkness is needed for the burst in gene expression and carotenoid accumulation in carrot roots (Fuentes et al. 2012,

Rodríguez-Concepción and Stange 2013). Little information is available about the effect of light on carotenoid biosynthesis in citrus fruit. In citrus species such as sweet oranges and mandarins, light exposure during fruit ripening generally enhances carotenoid accumulation and external fruit colour (Cronje et al. 2011, 2013). In contrast, SR grapefruit develop more intense red peel colouration when grown under low light conditions (field observations), indicating a different response to light levels compared to mandarins and oranges. To further understand the regulatory mechanisms of light on carotenoids biosynthesis in the lycopene-accumulating red grapefruit 'Star Ruby', carotenoid composition, and expression of carotenoid biosynthetic genes, were analysed in the peel of shaded fruit in comparison to those exposed to light under standard photoperiodic conditions. Additionally, ABA content, a downstream metabolite of the carotenoid pathway, was also analysed. We further explored possible changes in the structure of plastids during chloro- to chromoplast conversion, as well followed the expression of genes related to the transition of chloro- to chromoplasts such as those encoding enzymes like FIBs, Or and sHSPs.

MATERIALS AND METHODS

Plant material and treatments

Complete branches located inside and outside of the canopy of *Citrus paradisi* cv. 'Star Ruby' trees were tagged and colour changes during ripening followed in orchards located in the Northern (39º37'20"N, 0º33'38"W) and Southern (28°78'69"S, 20°65'19"E) hemispheres by using a Minolta CR-330 colorimeter. The orchard in the Northern hemisphere was located in Lliria (Valencia, Spain) with an average of 2660 hours of sunlight per year (heliophany) and a rainfall level of 454 mm (data from 1971 to 2000) and the orchard in Southern hemisphere was located in Kakamas with 3731 hours of heliophany and precipitation of 174.3 mm per year. For covering experiments, 105 fruits located outside of the canopy from 4 different trees of *Citrus paradisi* cv. 'Star Ruby' growing under commercial conditions in Lliria, Valencia (Spain) were tagged. Half of the tagged fruits were covered with black plastic bags in July at an immature green stage (55 ± 1 mm fruit diameter), before the initiation of natural degreening process, coinciding with 118 days after anthesis-DAA, and the bag end-bottom was left open to allow gas exchange. Three biological replicate samples of 5 fruit each were harvested as a starting point. In October, November and December, coinciding with mature green (187 DAA), breaker (224 DAA) and full colour (257 DAA) stages, three 5-fruit replicates for control fruit (Non covered, NC) and covered fruit (C) were harvested and the peel tissue excised, frozen in liquid nitrogen, ground to a fine powder and stored at -80 ºC until further analyses. At each harvest date, peel colour of whole fruit was measured using a Minolta CR-330 colorimeter on three areas of the equatorial plane of the fruit and expressed as the *a/b* Hunter ratio (Stewart and Wheaton 1972). The *a/b* ratio is negative for green fruit, the zero value corresponds to yellow fruit at colour break, and orange to red coloured fruit reflects a positive value.

Chlorophyll and carotenoid extraction

Peel pigments were extracted as previously described (Alquezar et al. 2008). The chlorophyll (a+b) content was determined by measuring the absorbance at 644 and 662 nm and

calculated according to the Smith and Benitez (1995) equations. After chlorophyll measurements the pigment ethereal solution was dried and saponified using a 10% methanolic:KOH solution. Carotenoids were extracted and the samples dried under N_2 and kept at -20 ºC until analysis. All procedures were carried out on ice under dim light to prevent possible photodegradation, isomerisation and structural changes of carotenoids.

Carotenoid analysis by HPLC

Carotenoid composition of each sample was analysed by HPLC with a Waters liquid chromatography system equipped with a 600E pump, a model 996 photodiode array detector, and Empower software (Waters). A C30 carotenoid column (250 × 4.6 mm, 5 µm) coupled to a C30 guard column (20 × 4.0 mm, 5 µm) (YMC Europe GMBH) was used. Samples were prepared for HPLC by dissolving the dried carotenoid extracts in $CHCl_3$: MeOH: acetone (3:2:1, v:v:v). A ternary gradient elution with MeOH, water and methyl *tert*-butyl ether (MTBE) was used for carotenoid separation as reported in previous work (Alquézar et al. 2008). The carotenoid peaks were integrated at their individual maxima wavelength and their content were calculated using the following calibration curves: β-cryptoxanthin (Extrasynthese) for α- and β-cryptoxanthin; lutein (Sigma) for lutein, neoxanthin and violaxanthin isomers; lycopene (Sigma); zeaxanthin (Extrasynthese) for zeaxanthin and antheraxanthin; α- and β-carotene (Sigma). Standards for calibration curves of phytoene, phytofluene and ζ-carotene were obtained from flavedo extracts of 'Pinalate' fruits, which accumulate large amounts of these compounds. Samples were extracted twice and each analytical determination was replicated at least twice. All operations were carried out on ice under dim light to prevent photodegradation, isomerisation and structural changes of carotenoids. To test for significant differences in carotenoids concentration between NC and C fruit at each harvest date a two tailed unpaired T student test was applied ($p \leq 0.05$). To test for significant differences in carotenoids concentration between NC and C fruits at each harvest date, a two tailed unpaired T student test was applied ($p \leq 0.05$).

Quantitative real time-PCR

Total RNA was isolated from the flavedo of fruit at each harvest date, using RNeasy Plant Mini Kit (Qiagen) and subsequently treated with DNase (DNA free, DNase treatment & removal, Ambion). The transcripts present in 2 µg of total RNA were reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen) in a total volume of 20 µL. One µL of a 5 times diluted first-strand cDNA, containing approximately 100 ng of cDNA, was used for each amplification reaction. Quantitative real-time PCR was performed on a LightCycler 480 instrument (Roche), using the LightCycler 480 SYBRGreen I Master kit (Roche). Reaction mix and conditions followed the manufacturer's instructions. The primers employed for the amplification of each gene are described in Table S1 (see Appendix S1 in Supporting Information). The protocol for all the genes analysed consisted of 10 min at 95 °C for pre-incubation, followed by 40 cycles of 10 s at 95 °C for denaturation, 10 s at 59 °C for annealing and 10 s at 72 °C for extension. Fluorescent intensity data were acquired during the extension time. Specificity of the PCR reaction was assessed by the presence of a single peak in the dissociation curve performed after the amplification steps. For expression measurements, LightCycler 480 Software release 1.5.0, version

1.5.0.39 (Roche) was used and expression levels relative to values of a reference sample were calculated using the Relative Expression Software Tool –REST (Pfaffl et al. 2002). Normalization was performed using the expression levels of the *actin* gene based on previous housekeeping selection (Alós et al. 2014). For all genes analysed, the reference sample was the expression value obtained in the peel of fruit harvested in July which was set at one. Results were expressed as the average of 4 independent replicates. To test for significant differences on transcript levels between NC and C fruit at each harvest date, a pair-wise fixed reallocation randomization test was applied ($p \leq 0.05$).

Abscisic acid quantification

Analysis was carried out by HPLC coupled to tandem mass spectrometry as described in de Ollas et al. (2013). Briefly, 0.4 g of frozen fruit peel was extracted in 5 ml of distilled water after spiking with 100 ng of [$^{2}H_{6}$]-ABA in order to avoid matrix effects and to assess recovery. After centrifugation at 4 °C, supernatants were recovered and pH adjusted to 3.0 with 30% acetic acid. The acidified water extract was partitioned twice against 3 mL of di-ethyl ether. The organic layers were combined and evaporated under vacuum. The dry residue was then re-suspended in a 10% MeOH solution by gentle sonication. The resulting solution was filtered and directly injected into a HPLC system (Waters Alliance 2695). ABA was separated in a reversed-phase Kromasil 100 C18 column (100 × 2.1 mm, 5 µm particle size; Scharlab) using a linear gradient of MeOH and water supplemented with 0.01% acetic acid at a flow rate of 300 µL min^{-1}. Quantification was performed with triple quadrupole mass spectrometer (Quattro LC, Micromass Ltd.) connected online to the output of the column through an orthogonal Z-spray electrospray ion source. To test for significant differences between NC and C fruit at each harvest date a two tailed unpaired T student test was applied ($p \leq 0.05$).

Transmission electron microscopy (TEM)

The peel tissue samples were manually cut in small squares of 5 mm long x 1 mm wide, fixed in modified Karnovsky fixative (0.5% glutaraldehyde, 2.5% formaldehyde in 0.1 M phosphate buffer pH 7.4). Specimens were then rinsed in 0.1 M PIPES buffer, postfixed in 1% buffered osmium tetroxide (1 h), rinsed in buffer, block stained in 2% aqueous uranyl acetate (20 min), dehydrated in an ethanol series and embedded in Spurr resin (Elektron Technology, Stansted, UK) in the normal way. Gold/silver ultrathin sections were then cut from suitable regions of the tissue and stained with Reynolds lead stain and viewed on a Hitachi H7000 transmission electron microscope equipped with a SIS Megaview III digital camera.

RESULTS

Fruit position on the tree canopy affects peel colour during ripening of Star Ruby grapefruit

In red lycopene-accumulating grapefruits, it was observed that the surface of the fruit covered by leaves or in contact with other fruit displayed a faster rate of degreening and also developed a more intense red colouration in the peel as compared with those directly exposed to sunlight (see Appendix S2 in Supporting information). Based on these field observations, we first

analysed the effect of natural fruit shading on peel colouration of SR grapefruits by comparing peel colour of fruits located on the outside of the tree canopy with those located inside and covered by leaves, from orchards of two distant locations in Northern (Lliria, Valencia, Spain,) and Southern (Kakamas, South Africa) hemispheres. Although the rate of fruit colouration was different in each location, probably due to differences in environmental and/or agronomic conditions, we consistently found that the red peel colour (higher *a/b* ratio) was more intense in fruits growing inside the canopy than outside of the tree (Fig. 2).

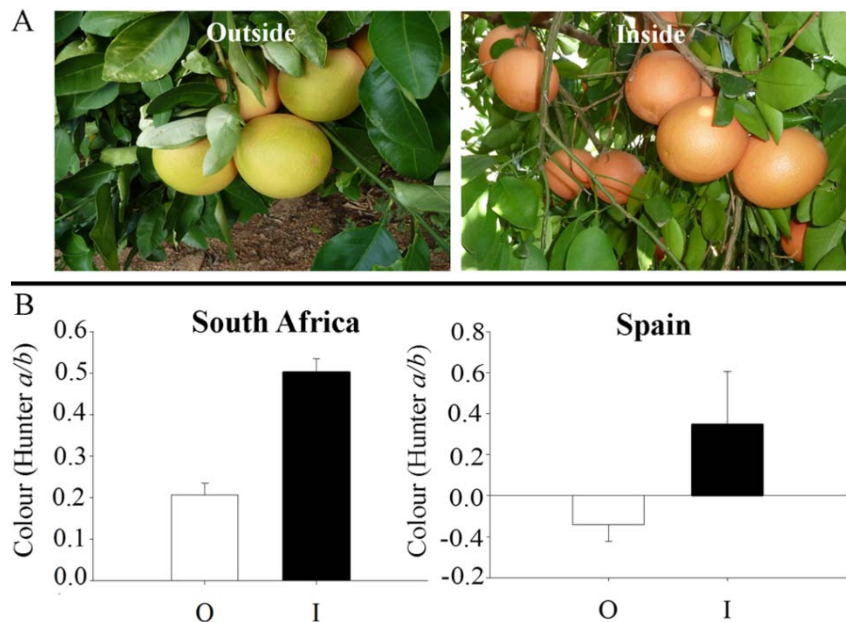

Figure 2. Effect of fruit shading on peel colour development in Star Ruby (SR) fruit. (**A**), Pictures representative of the external colour of SR fruit developed inside or outside of the tree canopy. (B) Colour (***a/b*** Hunter) in fruit developed inside (I) or outside (O) of the canopy harvested in May (270 days after anthesis) in South Africa and in November (260 days after anthesis) in Spain.

Effect of fruit bagging on peel colour, pigments composition and abscisic acid content in Star Ruby grapefruit

To study the effect of light avoidance on colour development and pigment composition in SR grapefruit, immature-green fruits (July) growing outside the tree canopy were covered by black plastic bags (C) and compared with non-covered (NC) fruit, exposed to normal photoperiodic conditions. Changes in peel colour and pigment concentration were determined periodically in both NC and C fruits throughout the ripening process, corresponding to mature green (October), breaker (November) and mature (December) stages for SR grapefruit (Alquézar et al. 2013). Covering SR fruit substantially accelerated peel colour development, resulting in significant differences in the *a/b* Hunter colour index compared to control fruit (Fig. 3). Three months after bagging (October), the peel of C fruit showed pink tones and reached an *a/b* ratio of 0.2 units, while in NC fruit the ratio was less than -0.2, corresponding to green-yellowish colouration. Differences in colour development between both treatments were maintained until the end of the ripening period, when

mature C fruits were fully red-coloured (*a/b* ratio of 0.45) whereas NC fruits were slightly yellow-orange with an *a/b* ratio 10 times lower (*a/b*=0.04; Fig. 3A).

In accordance with the rapid disappearance of green colour in C fruits, a faster chlorophyll (Chl) breakdown occurred. In the peel of NC fruit harvested in October, Chl content decreased by 27% whereas those covered fruit declined by 83%. In November, Chl concentration in the peel of NC fruits was still 33% of the initial values but in C fruit they were less than 10% of the original values. No Chls were detected in the peel of both NC and C fruit in December (Fig. 3B). Total carotenoid content in the peel of SR fruit at bagging time was 18.9 µg g^{-1} FW and remained almost constant until December in fruit exposed to normal photoperiodic conditions. In contrast, total carotenoid levels increased nearly 4-fold in the peel of C fruit in October to reach concentrations near 80 µg g^{-1} FW in November and December (Fig. 3C).

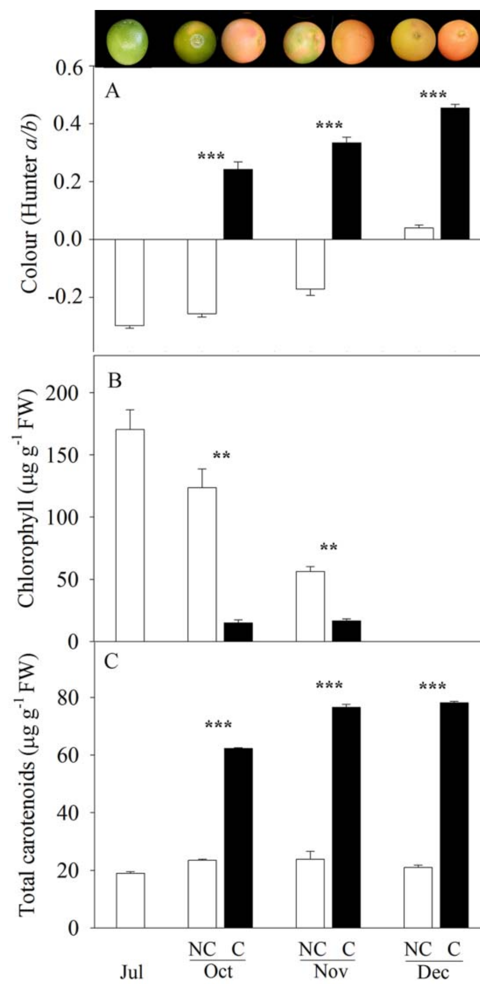

Figure 3. Effect of fruit bagging on (A) peel colouration (a/b Hunter), (B) total chlorophyll content and (C) total carotenoid content (µg g^{-1} FW) in Star Ruby grapefruits. Immature green fruit were bagged in July, and non-covered (NC) and covered (C) fruit were harvested in October (69 days after bagging, dab), November (106 dab) or December (139 dab), corresponding to mature green, breaker and full colour stages in NC fruit. Asterisks indicate significant differences between NC and C fruit at each harvest date (**p≤0.01 ***p≤0.001).

The effect of fruit bagging on the concentration of specific carotenoids was also determined (Table 1). The peel of immature green fruits (July) showed a carotenoid profile typical of chloroplastic tissue, lutein being the predominant carotenoid, followed by violaxanthin, β-carotene and neoxanthin, while linear carotenes represented only 6% of total carotenoids (Table 1). Although total carotenoid content experienced minor changes in NC fruit during ripening, individual carotenoids levels showed important modifications. Phytoene increased from 6 and 12-times in NC fruits in October and December, respectively, but it was always half of the content found in C fruits. Thus, linear carotenoids accounted for 37% and 75% of total carotenoids in NC fruits in the period from October to December (Table 1). Lutein, violaxanthin and neoxanthin contents decreased markedly during ripening (Table 1). β-carotene content in NC fruit remained almost constant until November, dropping to less than 1.0 μg g^{-1} FW in mature fruit (Table 1). Contrastingly, the peel of C fruit showed a massive accumulation of phytoene and phytofluene reaching values around 22 and 6 μg g^{-1} FW in December, respectively, when linear carotenes represented more than 90% of total carotenoids in the peel of C fruits (Table 1). The decline in lutein and other xanthophylls was faster in C fruits than in NC during ripening (Table 1). It is worth noting that the most striking difference in carotenoids composition between NC and C fruit was the drastic increase of lycopene in the peel of the latter. Lycopene concentration at time of bagging was almost negligible and increased to 35 and 45 μg g^{-1} FW in October and December, respectively, in C fruit (Table 1). Lycopene content at the same harvest dates was 30 and 49-times lower in NC fruits than in C ones (Table 1).

Table 1. Effect of fruit bagging on the composition of individual carotenoids (μg g^{-1} FW) in the peel of Star Ruby grapefruits. Immature green fruit were bagged in July, and non-covered (NC) and covered (C) fruit were harvested in October (69 days after bagging, dab), November (106 dab) or December (139 dab), corresponding to mature green, breaker and full colour stages in NC fruit. The data are means of three determinations ± SE.

	July	**October**		**November**		**December**	
Carotenoid	NC	NC	C	NC	C	NC	C
Phytoene	1.07±0.18	6.98±0.78	18.6±1.69	11.0±0.83	23.2±0.51	12.8±0.47	22.4±0.06
Phytofluene	-	0.35±0.01	4.03±0.61	1.03±0.14	6.13±0.24	1.82±0.12	6.20±0.31
ζ-Carotene	-	-	-	-	0.22±0.15	0.14±0.03	0.14±0.07
All-*E*-Lycopene	0.21±0.04	1.27±0.28	35.3±2.0	1.37±0.16	42.1±0.83	0.92±0.07	45.2±0.24
β-Carotene	2.01±0.03	2.00±0.02	2.16±0.05	2.03±0.27	3.61±0.01	0.69±0.01	3.10±0.21
α-Carotene	0.53±0.05	0.95±0.01	-	0.58±0.09	-	-	-
Zeaxanthin	0.38±0.06	0.46±0.02	-	1.00±0.18	-	0.27±0.01	-
Violaxanthin[a]	5.76±0.36	4.07±0.24	0.99±0.12	2.16±0.38	0.84±0.05	3.43±0.12	0.86±0.01
Neoxanthin[a]	1.39±0.12	-	-	-	-	-	-
α-Cryptoxanthin	0.16±0.01	-	-	(1)	-	(1)	(1)
Lutein	7.50±0.12	5.62±0.07	1.09±0.01	3.07±0.44	0.30±0.06	0.44±0.01	0.08±0.01

[a]Mix of isomers (1) values lower than 0.05 μg g^{-1} FW.

To investigate whether the effect of light avoidance on carotenoid content may also affect the accumulation of ABA, a direct downstream product of the carotenoid pathway, its content was determined in the peel of NC and C fruit. ABA peel content progressively increased with ripening of NC fruit, reaching a maximum in November. Under dark conditions, the increment in ABA was delayed and reduced, being at least 2-times lower in C than in NC fruits at all stages analysed (Table 2).

Table 2. Effect of fruit bagging on ABA concentration (ng g^{-1} FW) in the peel of Star Ruby grapefruits. Immature green fruit were bagged in July, and non-covered (NC) and covered (C) fruit were harvested in October (69 days after bagging, dab), November (106 dab) or December (139 dab), corresponding to mature green, breaker and full colour stages in NC fruit. The data are means of three independent replicates ± SE. Asterisks indicate significant differences between C and NC fruit at each harvest date (p≤0.05).

	ABA (ng g^{-1} FW)	
Date	NC	C
July	103 ± 12	--
October*	250 ± 14	83 ± 7
November*	742 ± 18	302 ± 25
December*	633 ± 17	187 ± 3

Effect of fruit bagging on transcript accumulation of carotenoid biosynthetic genes in the peel of Star Ruby grapefruit

To analyse whether changes in carotenoid composition induced by light were related to alterations in the expression of key genes of carotenoid biosynthesis, the relative transcript abundance of genes *PSY*, *PDS, ZDS, βLCY1, βLCY2* (a and b alleles) and *βCHX,* covering main steps of the pathway (Fig.1), were analysed in the peel of NC and C grapefruits. Transcript levels of most of the genes analysed (*PSY, PDS, ZDS1, βLCY1* and *βCHX*) was enhanced during fruit ripening and was notably higher in light-grown fruits, reaching maximum levels in November and decreasing thereafter (Fig. 4). The accumulation of transcripts of both *βLCY2* alleles, described as key regulators of the conversion of lycopene to β-carotene in citrus fruits (Alquézar et al. 2009, 2013), was highly stimulated by light throughout the whole ripening period (Fig. 4). A remarkable feature for all the genes analysed (except for the alleles 2 and 3 of *ZDS*), was the significant down-regulation in the peel of C fruits compared to NC (Fig. 4). Thus, the increase in the transcript levels in fruit from July to October was of 7 times for *PSY* and *ZDS1,* and 3 times for *PDS* in light exposed fruits whereas in C fruits these enhancements were only double or lower. On the other hand, transcription of genes which products are downstream lycopene, such as *βLCY1*, *βLCY2a, βLCY2b* and *βCHX* was similar in NC and C fruits until October, where after their relative transcript levels were again lower in C than in NC fruits (Fig. 4). *ZDS2* and *ZDS3* were the only genes whose expressions remained constant throughout the ripening process as well as between treatments, thereby suggesting that these two isoforms may not play a major role in the carotenoid changes taking place in grapefruit peel during ripening (Fig. 4).

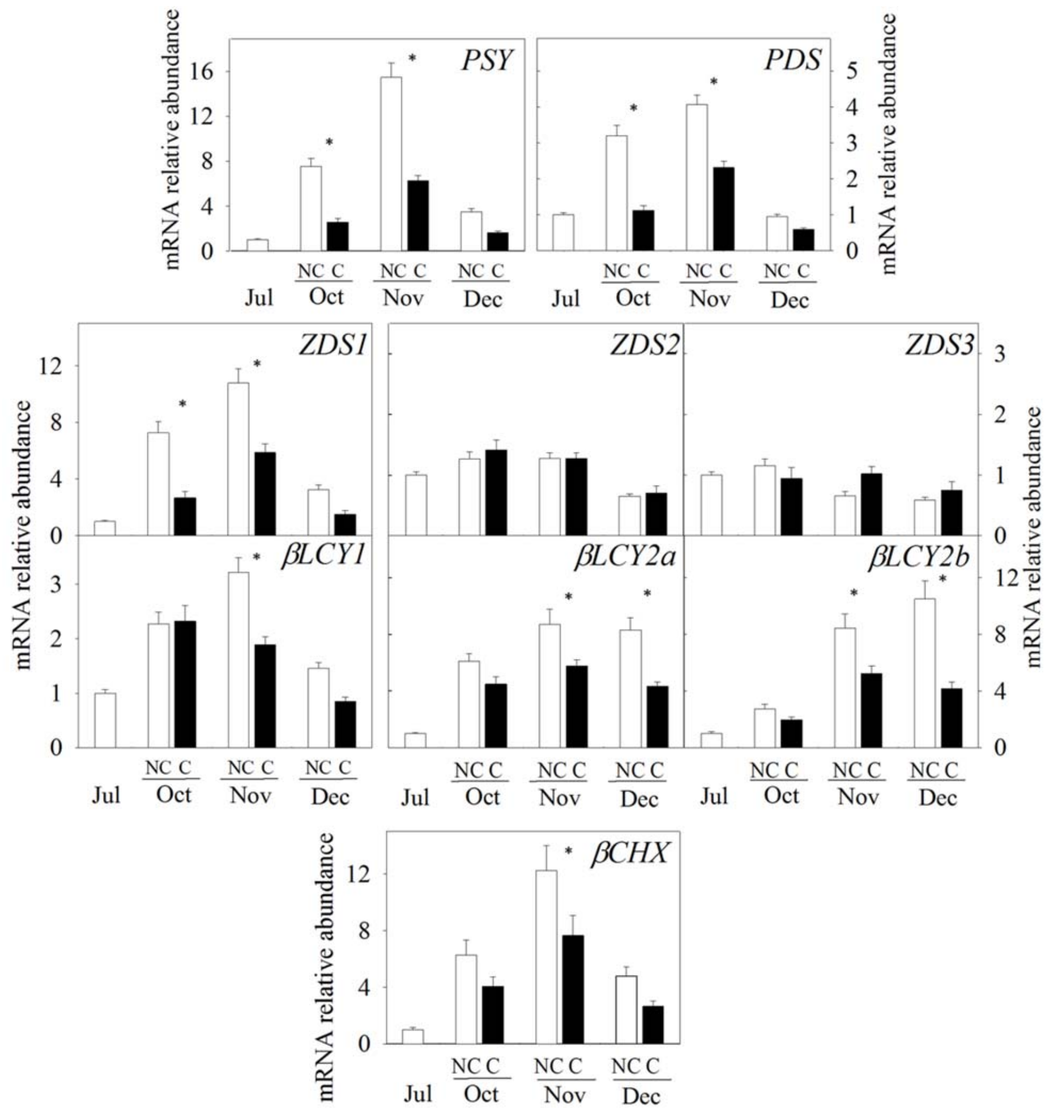

Figure 4. Effect of fruit bagging on the expression of carotenoid biosynthetic genes in the peel of Star Ruby grapefruits. Immature green fruit were bagged in July, and non-covered (NC) and covered (C) fruit were harvested in October (69 days after bagging, dab), November (106 dab) or December (139 dab), corresponding to mature green, breaker and full colour stages in NC fruit. The genes analysed were: *phytoene synthase* (*PSY*), *phytoene desaturase (PDS), ζ-carotene desaturase (ZDS1 ZDS2* and *ZDS3), lycopene β- cyclase 1(βLCY1), lycopene β- cyclase 2* (*βLCY2a* and *βLCY2b*) and *β-carotene hydroxylase (βCHX)*. The data are means ± SE of 4 replicates. Asterisks indicate significant differences between C and NC fruit at each harvest date ($p \leq 0.05$).

Effect of fruit bagging on transcript accumulation of key genes of the MEP pathway in the peel of Star Ruby grapefruit

We further investigated if the differences in carotenes accumulation induced by fruit bagging (Table 1) may be related to a differential transcriptional regulation of the MEP pathway. Analysis of transcript accumulation in the peel of NC and C fruits did not reveal substantial differences in expression of *DXS1*, *HDR* and *GGPPS* genes (Fig. 5). *DXS1* and *HDR* genes followed a similar expression pattern throughout ripening, with a transient induction in October and decreasing thereafter. The *GGPPS1* mRNA level increased progressively during ripening in both

NC and C fruits, although in the last mRNAs accumulation was lower in November (Fig. 5). In addition to the genes described above, a second isoform of *DXS* and *HDR* as well as six others of *GGPPS* gene were analysed. However, no differences were detected in the transcript levels between NC and C fruits (data not shown).

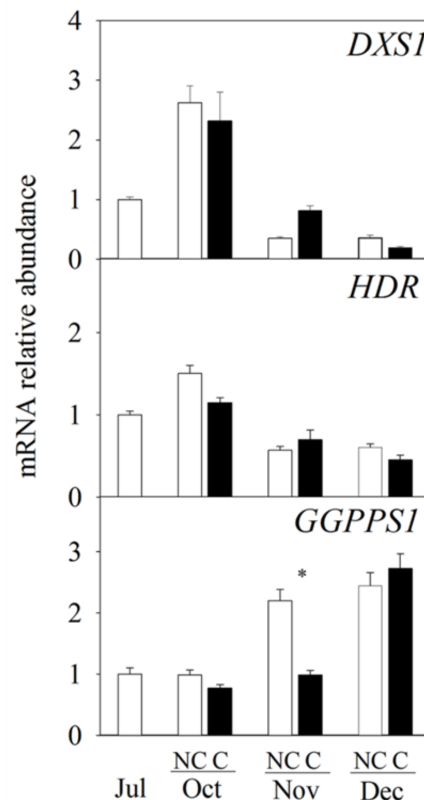

Figure 5. Effect of fruit bagging on the expression of the principal genes of the MEP pathway in the peel of Star Ruby grapefruits. Immature green fruit were bagged in July, and non-covered (NC) and covered (C) fruit were harvested in October (69 days after bagging, dab), November (106 dab) or December (139 dab), corresponding to mature green, breaker and full colour stages in NC fruit. The genes analysed were *1-Deoxy-D-xylulose-5-phosphate synthase (DXS1), hidroxymethylbutenyl diphosphate reductase (HDR)* and *geranyl geranyl pyrophosphate synthase (GGPPS1)*. No significant differences were detected between C and NC fruit at each harvest date ($p \leq 0.05$).

Effect of fruit bagging on transcript accumulation of genes involved in chromoplast substructures and differentiation in the peel of Star Ruby grapefruit

In order to elucidate if the accelerated rate of peel colour change and the massive carotenoid accumulation that occurred in C red grapefruit was associated with proteins involved in the differentiation of chromoplast and in carotenoid-sequestering structures, transcript levels of the genes fibrillin or plastid associated proteins (*FIB* or *PAP*), Orange (*Or*) and two sHSPs (*HSP20-4*, *HSP21*) were measured in the peel of NC and C fruits. A fibrillin (*FIB1-CitPAP*) has been previously described in citrus fruits (Moriguchi et al. 1998) and search on the *Citrus sinensis* genome database (www.phytozome.com) allowed the identification of a second putative FIB (FIB2, orange1.1g022196m.g) showing 31% of identity with FIB1 at amino acid level, and 71% identity with PAP-fibrillin of flax (*Linum usitatissimum;* Acc. JX_174449.1; Wang et al. 2012).

FIB1-CitPAP transcripts abundance slightly increased and *FIB2* remained constant with minor changes during ripening. However, in both genes no differences in the expression level between C and NC fruit were observed (Fig. 6).

sHPSs are chaperones with functions related to membrane stabilization or prevention of protein aggregation (Al-Whaibi 2011). Some of these proteins accumulated during fruit ripening and have been associated with carotenoid accumulation (Neta-Sharir et al. 2005). In this work two citrus members of sHSP potentially involved in chromoplast differentiation were identified: HSP20-4 and HSP21. Citrus *HSP20-4* presented a 83% identity with the orthologous from *Theobroma cacao* and 72% identity with *A. thaliana* class I *HSP* (AT1G07400; Theologis et al. 2000) and the *HSP21* of the flower *Cleome spinosa* (EU162609.1). *HSP21* is most likely the citrus orthologous of *HSP21* from tomato (38%) and the corresponding protein has been identified in the proteome of citrus fruit chromoplast (Zeng et al. 2011). Interestingly, both *HSP20-4* and *HPS21* genes showed a significant up-regulation (3 and 2 fold, respectively) in C fruit from July to October. At that time, transcript levels of both *HSP* genes in the peel of NC fruits were almost similar to that of immature green fruits (July; Fig. 6). In light exposed fruit, transcript levels of both genes increased in November and December, although with a different trend (Fig 6).

The Or protein has been associated with differentiation of chromoplast from non-coloured plastids in different plant tissues (Lu et al. 2006) and enhances accumulation of carotenoids (Li et al. 2001). An *Or* orthologous gene was identified in the sweet orange genome database (orange1.1g021509m) and its predicted protein presented a 76% identity with that of the *Or* gene from *Brassica oleracea* (ABH07405.1; Lu et al. 2006). Accumulation of *Or* transcripts was similar in the peel of NC and C grapefruit in October. The transcript level was progressively induced during ripening in NC fruits but remained stable in C grapefruits (Fig. 6).

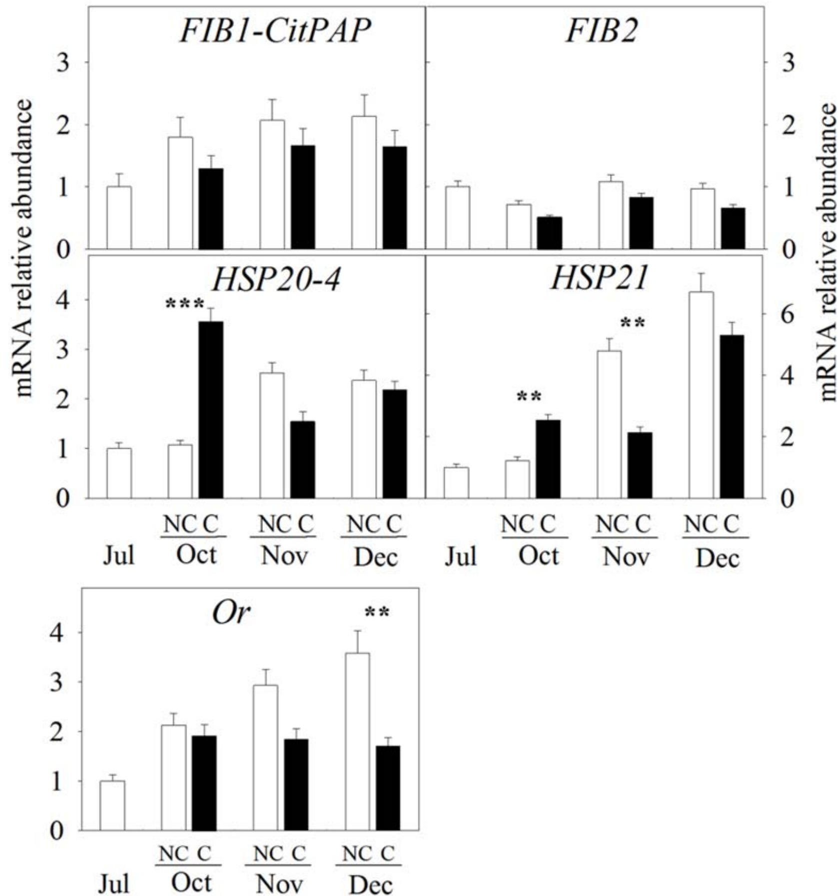

Figure 6. Effect of fruit bagging on the expression of fibrillins (*FIB1-CitPAP* and *FIB2*), Orange (*Or*) and small heat shock proteins (*HSP20-4* and *HSP21*) genes in the peel of Star Ruby grapefruits. Immature green fruit were bagged in July, and non-covered (NC) and covered (C) fruit were harvested in October (69 days after bagging, dab), November (106 dab) or December (139 dab), corresponding to mature green, breaker and full colour stages in NC fruit. Asterisks indicate significant differences between C and NC fruit at each harvest date (**$p \leq 0.01$ ***$p \leq 0.001$).

Effect of fruit bagging on chloro- and chromoplast ultrastructure in the peel of Star Ruby grapefruit

The peel of SR fruits in July presented the characteristic chloroplasts of photosynthetic tissues, with stacked grana formed by well-organized thylakoids, as well as starch grains (Fig. 7A). However, in October, important differences in the ultrastructure of the chromoplast were detected between NC and C fruit. NC fruit plastids still maintained well-organized thylakoids stacked into grana and some plastoglobuli could be observed (Fig. 7B) whereas the photosynthetic membranes of C fruit appeared to be disassembled with only a few grana and thylakoids visible (Fig. 7C). Moreover, carotenoid-storage structures like plastoglobuli and crystals, were observed after October inside the chromoplast of C fruit, whereas these structures were almost absent in NC fruit (Fig. 7B and C). In December, chromoplast of NC and C fruits contained round and even, highly osmiophilic plastoglobuli and the presence of lycopene crystals and electron dense uneven structures were only observed in C fruit (Fig. 7D and E). These electron dense osmiophilic structures were highly variable in size (ranging from 80 to 500 nm wide and from 80 to 800 nm

long). These structures were not found co-existing in the same plastid with typical plastoglobuli. The predominant structures in NC fruit were small, electron dense, spherical and even plastoglobuli together with the presence of non-photosynthetic membranes (Fig. 7D). It is important to note that no significant differences in chromoplast number or size were detected between NC and C fruit during ripening (data not shown).

Figure 7. Transmission electron microscopy images of chloro- and choromoplast in the peel of Star Ruby grapefruit in July (panel A) and in the peel of non-covered (NC) or covered (C) fruit harvested in October (69 days after bagging, dab) (panels B and C, respectively), and December (139 days after bagging) (panels D and E, respectively). **mm**, membranes; **m**, mitochondria; **Pg**, plastoglobule; **s**, starch granules; **t**, thylacoids.*lycopene crystals. Black arrows point to uneven electron dense structures detected only in covered fruit.

DISCUSSION

Colouration of citrus fruit is an important quality parameter directly affecting consumer preference and is strongly influenced by environmental and endogenous factors (reviewed by Kato 2012, Rodrigo et al. 2013). Light is recognized as one of the most important environmental factors stimulating citrus peel colouration during natural ripening. In oranges and mandarins it is frequently observed that fruits located inside the tree canopy or the internal side of fruits not exposed to light display lighter colouration than those directly exposed to light (Cronje et al. 2011, 2013). A similar stimulatory effect of light on carotenoid accumulation has been also described in tomato fruit (Azari et al. 2010). It is therefore paradoxical that the peel of SR grapefruit grown under natural shading, i.e. covered by leaves or inside the tree canopy, develops a more intense red colouration (Figs. 2 and S1, Supporting Information). Moreover, this observation in red grapefruit seems unrelated to differences in agronomical practices as occurred similar in Northern and Southern hemisphere orchards, with profoundly different environmental conditions. It is interesting that exceptions to this light-inductive effect on colouration have also been described in specific storage plant organs that usually grow under darkness, such as carrot roots (Fuentes et al. 2012, Rodríguez-Concepción and Stange 2013).

Light avoidance of SR grapefruit during ripening (by covering fruit with plastic bags at immature green stage) accelerated peel colour development compared to light-exposed fruit (Fig. 3). This effect was accompanied by a higher rate of chlorophyll degradation, massive accumulation of carotenes and a reduction of xanthophylls as well as downstream products such as ABA (Fig. 3, Tables 1 and 2). Moreover, total carotenoid content remained almost constant in the peel of fruits exposed to normal photoperiodic conditions whereas fruit grown under darkness registered a 4-fold increment with the most noticeable change occurring at an early stage of fruit ripening, from July to October (Fig. 3, Table 1). Despite the absence of changes in total carotenoids in light-grown fruits, the carotenoid profile was profoundly affected, showing a significant increase in the linear carotenes phytoene and phytofluene and, to a lesser extent, in lycopene (Table 1). Absence of light promoted an increased accumulation of lycopene which was also accompanied by accumulation of phytoene and phytofluene (Table 1). Therefore, in October, linear carotenes represented more than 90% of total carotenoids in C fruit, compared to only 37% in NC (Table 1). This dark-induced alteration in carotenoid profile suggests the accentuation of the bottleneck at lycopene cyclization level in the carotenoid pathway that was described to exist in the red grapefruit compared to oranges or white grapefruits (Alquézar et al. 2009, 2013). In the present study, the absence of light seems to potentiate this blockage as suggested by the higher concentration of lycopene and upstream carotenes, and the lower levels of downstream metabolites (xanthophylls and ABA) in C fruit as compared to NC fruit (Tables 1 and 2).

This increase in linear carotenes in the peel of C SR fruits was only partially mirrored by changes in the transcript accumulation of carotenoid biosynthetic genes. The most important changes in carotenoid composition occurred between July and October, when *PSY*, *PDS* and *ZDS* transcripts levels were higher in NC fruit than in C fruit (Fig. 4). In light growing conditions, the expression of the carotenoid biosynthetic genes, mainly *PSY, PDS*, *ZDS1* and *βLCY2,* was markedly up-regulated in the peel of SR fruit, which is similar to that reported in other fruits like tomato (Gupta et al. 2014) and vegetative tissue of *Arabidopsis thaliana* (Toledo-Ortiz et al. 2010).

Despite this fact, it is worth noting that the relative low transcripts levels of key carotenoid biosynthetic genes under darkness may be enough to allow a major lycopene accumulation in the peel of C fruit (Table 1). These results indicate that the activity of upstream steps in C fruit might be sufficient to provide an important flux of intermediates into the pathway and therefore, an elevated lycopene production. However, the lycopene β-cyclase activity should not be enough to effectively cyclize this carotene, which is supported by the lower downstream products (xanthophylls and ABA) found in C fruit (Table 1 and 2). The βLCY2 enzyme has been described as a limiting step in the carotenoid biosynthetic pathway in SR grapefruit due to reduced gene expression that contributes to lycopene accumulation in the pulp compared to other *Citrus* species or grapefruit varieties (Alquézar et al. 2009, 2013, Costa et al. 2012, Mendes et al. 2011). It may therefore be suggested that the reduced increase in the transcript accumulation of *βLCY2* in dark-grown fruits may contribute to enhance lycopene content in this tissue.

Light did not affect mRNA levels of the key genes from the MEP pathway in SR grapefruit (Fig. 5), the main source of precursors for carotenoid biosynthetic pathway. It has been reported that light has a positive effect on the MEP pathway (Botella-Pavía et al. 2004) however, in SR grapefruit light does not seem to play an important role in the transcriptional regulation of these genes (Fig. 5). Despite the absence of differences in transcript levels of genes from the MEP pathway it cannot be ruled out the possible existence of greater substrate availability under dark conditions due to reduced competition with other biosynthetic pathways. For example, it is well established that under dark conditions, chlorophyll degradation is accelerated but chlorophyll biosynthesis is also impaired; carotenoids and chlorophyll biosynthetic pathways share common precursors (Hemmerlin et al. 2012) thus the reduced demand of substrates for chlorophyll biosynthesis could result in greater availability for carotenoid biosynthesis when light levels are reduced.

The peel of shaded fruits showed an accelerated conversion of chloroplasts into chromoplast compared to those growing under light regime. This situation imposed remarkable changes in plastids ultrastructure and favoured the early appearance of structures to accumulate carotenoids (Fig. 7). Hence, the increase in the capacity to sequester newly synthesized carotenoids concomitant with the development of new biosynthetic sites in chromoplast, the plastoglobuli (Ytterberg et al. 2006), could be explaining the massive carotenoid accumulation in C SR fruit. Plastoglobuli have been suggested to be involved in chromoplast biogenesis being also an active site for carotenoid biosynthesis (Ytterberg et al., 2006) and accumulation (Nogueira et al., 2013). Thus, the earlier presence of plastoglobuli in C fruit compared to NC could be contributing to the higher carotenoid content in C fruit (Fig. 7). The importance of adequate structures for carotenoid biosynthesis and accumulation has been demonstrated since defects in plastid development results in impaired accumulation of carotenoids. Thus, a notable increase in storage capacity may explain the enhancement in the accumulation of carotenoids by enhancing sink-strength in different organs (Kim et al. 2013, Li et al., 2001, Li and Van Eck 2007). An illustrative example of how light can directly affect carotenoid accumulation through modification of plastids ultrastructure has been described in carrot roots developed under light. In this tissue, differentiation of leucoplasts into chloroplasts instead of chromoplast prevented the typical burst in carotenoid accumulation that occurs under normal conditions (darkness) (Rodriguez-Concepcion and Stange 2013). These results

agree with the idea that the formation of chromoplast is an important 'driving force' regulating carotenoids accumulation in plants. It has also been shown that the heterologous overexpression of the *Or* gene, which is related to chromoplast differentiation in several plant species, leads to massive accumulation of carotenoids without affecting the transcript levels of main biosynthetic genes (Kim et al. 2013, Li et al. 2001). Similar results were obtained in rice callus, where chromoplast differentiation is triggered either by a direct expression of *Or* gene or by increasing the flux through the carotenoid pathway (Bai et al., 2014). Thus, organelle biogenesis is a determinant of the storage capacity of the plastids and can directly affect carotenoid accumulation by providing a larger sink (Cazzonelli and Pogson 2010). From our results, it is difficult to establish whether abnormal carotenoid and lycopene accumulation in C SR fruits is linked to altered chromoplast differentiation as a result of reduced light or alternatively, may be a consequence of a modification in the balance of carotenoid metabolism.

The transcript accumulation of genes coding for proteins that are induced during differentiation of chloro- to chromoplast, such as of *FIB*s, *Or* and small *HSP*s genes (Lu et al. 2006, Neta-Sharir et al. 2005), have also revealed differences between NC and C fruits. Among the genes investigated, only *HSP20-4* and *HSP21* were specifically stimulated in dark-grown fruits (Fig. 6) and, interestingly, this was coincident with advanced chromoplast differentiation of those fruit in October (Fig. 7C). Nevertheless, the function of these HSPs in SR peel appears to be related to chloro- to chromoplast conversion and not to the massive lycopene accumulation in these organelles, since similar transcript levels were detected in both fruits in November. This coincides with the onset of chromoplast differentiation in light-grown fruits (Fig. 7), which presented a relative low content of lycopene (Table 1). Recent work described the relevance of these proteins as cofactors in the targeting of proteins to the plastid outer envelope to favor plastid differentiation and development (Kim et al. 2011). Similarly, *HSP21* was also described to be induced in tomato fruit during ripening (Neta-Sharir et al. 2005) and its transcript abundance was higher in cassava genotypes that accumulated high amounts of β-carotene compared to the white varieties, suggesting a specific role of this protein on carotenoid sequestration (Carvalho et al., 2012). Regarding citrus fruit, these sHSPs have also been reported to be induced in lycopene-accumulating citrus mutants like 'Cara Cara' sweet orange (Pan et al. 2012) and were suggested to be relevant to the ripening process, as it is of greater abundance in the plastid proteome of sweet orange (Zeng et al. 2011). FIBs proteins were also proposed to play a role in maintaining structural integrity of the plastoglobuli and their overexpression in tomato fruit, leading to an increase in carotenoid accumulation and to a new cluster arrangement of the plastoglobuli (Simkin et al., 2007). However, chloro- to chromoplast conversion in SR grapefruit does not seem to be related to fibrillin mRNA levels (*FIB1-CitPAP* and *FIB2*) (Fig. 6), thereby opposing what has been described in tomato and pepper fruit (Simkin et al. 2007). In contrast, in the lycopene-accumulating orange mutant 'Hong Anliu', a higher expression of *CitPAP* was described compared to the parental variety (Pan et al., 2009), which was not observed in SR grapefruit (Fig. 6) and thus suggesting the existence of different mechanisms between oranges and grapefruits in the metabolic process leading to lycopene accumulation. Similarly, the transcript level of *Or* gene showed no relevant changes during ripening in the absence of light, although in fruits grown in light conditions its expression showed an increment during ripening (Fig. 6). Therefore its function maybe related to chromoplast division

and mediation in proteins interactions (Li et al. 2001, Lu et al. 2006) that may be specially required under light conditions.

The experimental system used in this work revealed that high lycopene accumulation in SR fruit grown under darkness is tightly associated with changes involving the acceleration of chromoplast ultrastructural differentiation, resembling the process of green tissue senescence. In this sense, a premature chromoplast differentiation may provide a higher biosynthetic capacity and sink for carotenoid accumulation without necessary involving major transcriptional changes in the carotenogenic genes. The transcripts accumulation induction of most upstream carotenoid biosynthetic genes that normally occur in citrus fruit during maturation (Rodrigo et al. 2004) was also observed under dark conditions with maximal expression in November, which may sustain carotenoids accumulation in a global scenario of high substrate availability and an increased storage capacity in C fruits. A similar situation has been described in the regulation of carotenogenesis in carrot roots, where the massive accumulation of carotenoids in dark-grown roots was only partially correlated with the expression of carotenogenic genes, suggesting that additional mechanisms are also crucial in determining carotenoid content and composition in this organ (Rodríguez-Concepción and Stange 2013). It is important to emphasize the notion that in SR grapefruit lycopene cyclization is highly reduced due to a lower expression and less active *βLCY2* enzyme in red grapefruit compared to other citrus cultivars (Alquézar et al. 2009; Alquézar et al., 2013). Hence, in this scenario, reduced expression of all *βLCYs* in the peel of dark-grown fruits (Fig. 4) may potentiate the bottleneck at the lycopene cyclization step in the pathway promoting massive accumulation of lycopene and upstream carotenes. This blockage of lycopene cyclization in fruit developing in the absence of light is further supported by the reduced amounts of xanthophylls and, more specifically, of the downstream phytohormone ABA. Thus, in SR fruit light signals seem to regulate carotenoid accumulation at the molecular and structural level by influencing both biosynthetic capacity and sink strength. Furthermore, this work supports with the hypothesis that carotenoid accumulation can be boosted by triggering the synthesis of a plastid deposition sink to allow their enhanced biosynthesis and storage.

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SUPPLEMENTARY MATERIAL

Appendix S1

Table S1. Sequences of the primers used for real-time qPCR. The genes analysed are *1-Deoxy-D-xylulose-5-phosphate synthase* (*DXS1*), *β-lycopene cyclase 1 (βLCY1)*, *β-lycopene cyclase 2* (*βLCY2a and βLCY2b*), *β-carotene hydroxylase (βCHX), fibrillins or plastoglobulins (FIB1-CitPAP* and *FIB2), geranyl geranyl pyrophosphate synthase (GGPPS1), heat shock proteins 4 and 21 (HSP20-4* and *HSP21), hidroxymethylbutenyl diphosphate reductase* (*HDR*), *Orange* (*Or*) *phytoene synthase* (*PSY*), *phytoene desaturase (PDS),ζ-carotene desaturase (ZDS1, ZDS2, ZDS3), ζ-carotene isomerase (ZISO)* and *actin*.

Gene	Primer	Orientation[a]	Primer sequence (5'-3')
PSY	MJ134	S	GGTCGTCCATTTGATATGCTTG
	MJ135	AS	CCTAAGGTCCATCCTCATTCCT
PDS	MJ368	S	TCCCTTCTAAGTGTGTATGCC
	MJ369	AS	TGCAAGCTCCTTCATTGTAGC
ZDS1	MJ277	S	ACAATCTGTTTGAGGCGCAG
	MJ278	AS	CATAGGTATTGGAAACCCTTACTCC
ZDS2	MJ279	S	CTTTTACTGATGAACTAAGTAAATGGTTGT
	MJ280	AS	AAAATTGATTATCTCATTGGAATTACATGGA
ZDS3	MJ281	S	CATGGATAGTATGGGATGAGCAATCTG
	MJ282	AS	AATGAACAAACGTTTGTGTAGAGGTC
βLCY1	MJ136	S	GAACCAGGAGCTTAGGTCTG
	MJ137	AS	GCTAGGTCTACAACAAGGCC
βLCY2a	MJ426	S	GAGCAAGTCTCATCGCGTCATAGTG
	MJ427	AS	ACTTTAGCCTTATGAAACTTAACTCCATTTG
βLCY2b	MJ430	S	GCAAGTCTCATCGCGTCATGGTA
	MJ431	AS	ACTTTAGCCTTATGAAACCTAACGCCATTTA
βCHX	MJ126	S	GGCTCATAAAGCTCTGTGGC
	MJ127	AS	CCAGCACCAAAACAGAGACC
DXS1	*DXSF*	S	CGTGTTTTCAACACACCTGACG
	DXSR	AS	AAGCCCCGAAGTCTTCCTCAT
HDR	MJ462	S	GGTAAATAAGGGTGATGTTGTGGTTTTGC
	MJ466	AS	GAAGTATAGTCTCCCTTCTTGTGCTTCTC
GGPPS1	MJ478	S	CCGAGGTCAGCCCTCAAACC
	MJ479	AS	CTCAGGCACGAGATGGGGG
FIB1-CitPAP	MJ642	S	GGTGGCAGAGGAGGAGAG
	MJ643	AS	GGCATTTAGCAGAGTTAAGGC

FIB2	MJ518	S	GGAGCTCCCGCCGCTTAC
	MJ519	AS	GTTGGATTGAGCCGCTCAAGAC
Or	MJ652	S	GATGTTGATGTGTTGCGGCGG
	MJ653	AS	AAGTCCTGCACTGTTTCAGGACC
HSP20-4	MJ639	S	TCCGGTTATTCGCCTGCGC
	MJ543	AS	TGACCGCTTATCTGAAGCACCC
HSP21	MJ650	S	GGGGAAGAAGAAGAGTGGCC
	MJ651	AS	TGTCGACGATTTTGGCAGTGG
ACTIN	ACTINF	S	TTAACCCCAAGGCCAACAGA
	ACTINR	AS	TCCCTCATAGATTGGTACAGTATGAGAC

[a] S, sense; AS, antisense

Appendix 2

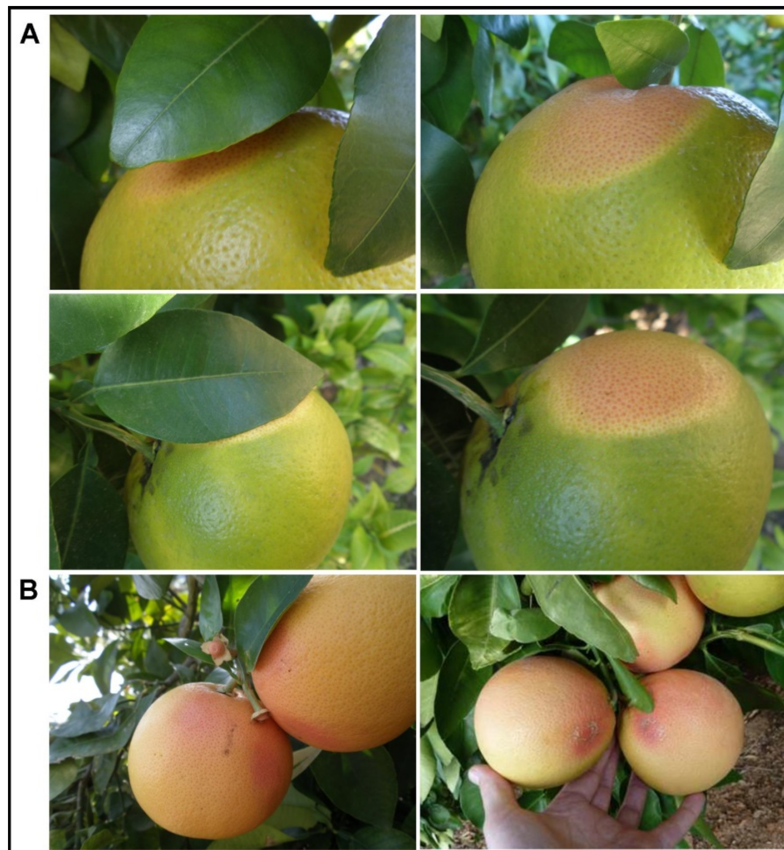

Figure S1. Effect of natural shading on peel colouration of SR fruit. Leaf shading (A) or natural contact between fruit (B) caused an evident enhancement of red fruit peel colouration.

3.5 CAPÍTULO V.

Exploring the diversity in *Citrus* fruit coloration to decipher the relationship between plastid ultrastructure and carotenoid composition

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Abstract

The *Citrus* genus displays a wide range in fruit colouration due to differences in carotenoids, however, how this diversity is related and may contribute to plastid differentiation and ultrastructure is currently unknown. To that end, carotenoid profile and plastid ultrastructure were compared in peel and pulp of three sweet oranges: the ordinary orange-coloured Navel rich in β,β-xanthophylls, the yellow Pinalate mutant with an elevated content of colourless carotenes and reduced β,β-xanthophylls, and the red-fleshed Cara Cara with high concentration of colourless carotenes and lycopene in the pulp; and two grapefruits: the white Marsh with low carotenoid content and the red Star Ruby, accumulating upstream carotenes and lycopene. The most remarkable differences in plastid ultrastructure among varieties were detected in pulp at full-colour, coinciding with major differences in carotenoid composition. Accumulation of lycopene in Cara Cara and Star Ruby pulp was associated with the presence of needle-like crystals while high content of upstream carotenes in Pinalate pulp was related to the development of a novel plastid type with even round globules. The presence of plastoglobuli was linked to phytoene and xanthophylls accumulation, suggesting these structures as the main sinks for these pigments. Peel chromoplast were richer in membranes compared to pulp chromoplast, reflecting their different biogenesis. In summary, differences in carotenoid composition and accumulation of unusual carotenoids are mirrored by the development of diverse and novel chromoplast types, revealing the plasticity of these organelles to rearrange carotenoids inside different structures to allow massive accumulation and their chemical stability.

INTRODUCTION

Citrus fruit is the first tree-crop in the world in terms of production and colour is one of the most important attributes of fruit quality. Carotenoids are the pigments responsible for the colour singularities of *Citrus* fruit and its development during ripening involves coordinated changes in chlorophyll degradation and biosynthesis and accumulation of carotenoids in both peel and pulp tissues. Carotenoids, in addition to the relevant functions in plants (Britton 2008), are also important nutritional components of the human diet, since some of them are precursors of vitamin A and present a relevant antioxidant capacity, playing then a role in the protection against degenerative and chronic diseases (Berman et al. 2014). Due to these relevant functional properties and their importance in the organoleptic quality of citrus fruit, carotenoid content and composition and their metabolic regulation in the peel and pulp of different citrus species and varieties have been extensively studied over decades (reviewed in Kato et al. 2012 and Rodrigo et al. 2013).

Carotenoids are synthetized in the plastids being the most representative the chloro- and chromoplasts, organelles that in addition to their role in essential metabolic pathways, also contribute to the storage of these pigments in many different specialized structures (Ljubesic et al. 1991; Li and Yuan 2013; Nogueira et al. 2013). Carotenoids in chloroplasts are mainly located in photosynthetic membranes in the form of chlorophyll-carotenoid-protein complexes involved in light harvesting and photoprotection (Vishnevetsky et al. 1999). Chromoplasts are defined as plastids which are photosynthetically inactive but have developed unique mechanisms to synthesize and accumulate large amounts of carotenoids which content appears to be determined not only by the rate of biosynthesis and degradation, but also by the capacity of developing stable storage sink structures (Li and Yuan 2013). During ripening of citrus fruit peel, the transition from chloroplasts into chromoplasts involves changes in structure, morphology and composition. These changes implicate a controlled breakdown of chlorophyll, a decrease in chloroplastic-type carotenoids content, thylakoid membranes disassembly, starch unfolding and the appearance of new sites for carotenoids biosynthesis and accumulation, such as plastoglobuli (Gross 1987). Plastoglobuli are lipid bodies containing different carotenoid biosynthetic enzymes among other proteins and constitute a relevant structure where carotenoids are synthetized, sequestered and stored (Ytterberg et al. 2006; Bréhélin et al. 2008; Li and Yuan 2013; Nogueira et al. 2013). Plastoglobuli are inside the chromoplasts of different carotenoid-containing fruits (Vasquez-Caicedo et al. 2006; Schweiggert et al. 2011; Nogueira et al. 2013), including citrus (Thomson 1966; Burns et al. 1992; Zeng et al. 2011; Cao et al. 2012), and increase in size and number during ripening (Ljubesic et al. 1991). Other structures have been described to coexist with plastoglobuli in the chromoplasts and the relative abundance of each type leads to the classification as globular, tubular, membranous and crystalloid chromoplasts (Sitte et al. 1980; Egea et al. 2010). Moreover, the development of sub-chromoplast structures seems to be related to the type of carotenoids that are sequestered (Ljubesic et al. 1991; Nogueira et al. 2013) and these structures may act also as a physical barrier influencing the bioaccessibility of carotenoids from fruits and vegetables (Jeffery et al. 2012).

Citrus fruits are one of most complex sources of carotenoids, with a large diversity of these compounds among the different species and cultivars in terms of types and amounts (Kato et al. 2004; Fanciullino et al. 2006; Xu et al. 2006; Kato 2012; Rodrigo et al. 2013). Interestingly, a common feature in mature citrus fruit is that carotenoid concentrations are usually higher in the

peel than in the flesh (juice vesicle) and most of the citrus species and varieties display a different profile in both tissues, suggesting differential regulation of biosynthesis and accumulation of these pigments in these fruit tissues (Tadeo et al., 2008). Moreover, differences in chromoplast structures have been also identified among the fruit of different citrus varieties what could be associated with the diversity of carotenoid complement and concentration, as has been shown in fruit from other species (Egea et al. 2010; Li and Yuan 2013). Pioneer electron microscopy studies about chromoplasts in citrus fruit revealed the presence of electron-dense plastoglobuli (400-800 nm) combined with membranes in mature Valencia oranges (Thomson 1966). By contrast, two types of chromoplasts were observed in flavedo (outer colured layer of fruit peel) cells of ripe pummelo (*Citrus grandis*), one of the citrus ancestral species: a typical one containing large plastoglobuli and vesicles, and an unusual type containing plastoglobuli but also long concentric achlorophyllous membranes, that predominated numerically (Gross et al. 1983). Plastoglobuli were also reported to be present in flavedo chromoplasts of Satsuma mandarin (Shimokawa et al. 1978), grapefruit (Gross 1987) and lemon (Ljubesic 1984). The chromoplasts from the pulp of the red Star Ruby grapefruit were described as sparse and exclusive of the outer cells of the juice sacs and seemed to be dominated by electron-transparent vesicles and electron-dense background material (Jeffery et al. 2012). Cao et al. (2012) have classified the chromoplasts of the peel of Marsh grapefruit, Cara Cara orange and Sunburst mandarin as globular, due to an important presence of plastoglobuli.

The relationship between the type of sub-chromoplast structures and the carotenoid content and composition during ripening of citrus fruit is not well understood and whether the accumulation of specific carotenoids may be a key factor determining the differentiation and development of particular carotenoid-containing structures in the chromoplast is currently unknown. Therefore, taking advantage of the great diversity in fruit colouration, and carotenoid singularity and composition among *Citrus*, the main objective of the present study has been to investigate the changes in chromoplast ultrastructure during ripening in relation to the carotenoid complement in peel and pulp tissues of fruits exhibiting contrasting colouration. To that end, fruits from the ordinary Navel sweet orange (*Citrus sinensis* L. Osbeck), with the typical standard orange colouration, which accumulates β,β-xanthophylls, mainly 9-*(Z)*-violaxanthin, as well as lower amounts of colourless carotenoids and apocarotenoids C_{30} (Oberholster et al. 2001; Rodrigo et al. 2004; Rodrigo et al. 2013), were selected and compared with two sweet orange spontaneous mutants, the yellow Pinalate and the red-pulp Cara Cara. Pinalate is a unique mutant with a distinctive pale-yellow colouration in peel and pulp of mature fruit accumulating significant amounts of early linear carotenes: phytoene, phytofluene and ζ-carotene isomers while the proportion of β,β-xanthophylls is extremely reduced, envisaging then a blockage at the desaturation of ζ-carotene (Rodrigo et al. 2003). Similarly, a natural mutation derived from the Navel sweet orange have led to the appearance of the red-fleshed Cara Cara (Xu et al. 2006; Alquézar et al. 2008). Cara Cara orange peel contains similar levels of 9-(*Z*)-violaxanthin than its parental Navel but a higher content of the colourless phytoene, while the flesh displays a reddish tonality due to the presence of lycopene, which is a very uncommon carotene in citrus fruit (Xu et al. 2006; Alquézar et al. 2008; Zeng et al. 2011). Among the genus *Citrus*, grapefruit (*Citrus paradisi*) is one of the species with higher diversity in external and internal fruit colouration, ranging from red or pink to pale yellow (Gmitter, 1995). Among the so called white grapefruits, the Marsh grapefruit is

one of the largest cultivated and consumed worldwide and contains only minute amounts of phytoene and violaxanthin in peel and pulp, whereas the red grapefruit Star Ruby, is described as one of the varieties with a more intense red colouration (Gmitter 1995), accumulating large amounts of carotenoids, mainly phytoene, phytofluene and lycopene in both tissues (Xu et al. 2006; Cao et al. 2012; Alquezar et al. 2013). Thus, in this study we have performed the comparison between the white Marsh and the red Star Ruby grapefruits, in order to establish whether the presence of lycopene affect plastid ultrastructure and also the comparison among three oranges: the yellow Pinalate and the red-fleshed Cara Cara orange mutants and the ordinary orange-coloured Navel, to establish if the altered carotenoid content and composition in the mutants is also influenced by plastid differentiation and sub-structures development.

MATERIALS AND METHODS

Plant material and treatments

Fruit of the ordinary Navel orange (*Citrus sinensis* L. Osbeck), Cara Cara and Pinalate sweet orange mutants, and the white Marsh and the red Star Ruby grapefruit (*Citrus paradisi* Macf.) were harvested from adult trees located at the Spanish Citrus Germplasm Bank (IVIA, Moncada, Valencia, Spain) subjected to the same environmental conditions and agronomical practices. For all varieties fruits were harvested at two ripening stages, breaker and full colour. At least 3 replicate samples of 10 fruits were harvested at each sampling date. Fruits were delivered to the laboratory, selected by size uniformity and those free of any defect were used for colour measurements. Peel and flesh colour of whole fruit was measured using a Minolta CR-330 colorimeter. Colour was expressed as the *a/b* Hunter ratio where the *a/b* ratio is negative for green fruit, the zero value corresponds to yellow fruit and is positive for orange coloured fruit (Stewart and Wheaton 1971). For pigments analysis, flavedo (external coloured portion of the peel) and pulp tissues were excised and frozen in liquid nitrogen, ground to a fine powder and stored at -80 ºC until analysis.

Chlorophyll and carotenoid extraction

Flavedo and flesh pigments were extracted as previously described (Alquézar et al. 2008). The chlorophyll (a+b) content in breaker fruit was determined by measuring the absorbance at 644 and 662 nm and calculated according to the Smith and Benitez equations (Smith and Benitez 1955). After chlorophyll measurements the pigment ethereal solution was dried and saponified using a 10% methanolic: KOH solution. Carotenoids were extracted and samples dried under N_2 and kept at -20 ºC until analysis. All procedures were carried out on ice under dim light to prevent possible photodegradation, isomerisation and structural changes of carotenoids.

Carotenoid analysis by HPLC

Prior to HPLC analysis, carotenoid extracts were dissolved in acetone and incubated overnight at -20º C to precipitate sterols which could interfere in the carotenoid analysis and subsequently dried under N_2. Carotenoid composition of each sample was analysed by HPLC with a Waters liquid chromatography system equipped with a 600E pump and a model 2998 photodiode

array detector, and Empower software (Waters). A C30 carotenoid column (250 × 4.6 mm, 5 µm) coupled to a C30 guard column (20 × 4.0 mm, 5 µm) (YMC Europe GmbH) was used. Samples were prepared for HPLC by dissolving carotenoid extracts in $CHCl_3$: MeOH: acetone (3:2:1, v:v:v). A ternary gradient elution with MeOH, water and methyl *tert*-butyl ether (MTBE) was used for carotenoid separation reported a previous work (Alquezar et al. 2008). Carotenoids were identified by comparison of the spectra and retention time with those of authentic standards, when available, or by matching the observed versus literature spectral data and retention time under identical chromatographic conditions (Britton 1995; Rodrigo et al. 2004). The carotenoid peaks were integrated at their individual maxima wavelength and their content were calculated using calibration curves of β-cryptoxanthin (Extrasynthese), lutein (Sigma) for lutein, neoxanthin, violaxanthin isomers, zeaxanthin (Extrasynthese), α- and β-carotene (Sigma) and lycopene (Sigma). Standards of phytoene, phytofluene and ζ-carotene for quantification were extracted from flavedo of Pinalate fruits, which accumulate large amounts of these compounds (Rodrigo et al. 2003), and afterward purified by thin layer chromatography (Pascual et al. 1993). Samples were extracted twice and each analytical determination was replicated at least twice. All operations were carried out on ice under dim light to prevent photodegradation, isomerisation and structural changes of carotenoids.

Transmission electron microscopy (TEM)

The peel and pulp tissue samples were manually cut in small squares of 5 mm long x 1 mm wide, fixed in modified Karnovsky fixative (0.5% glutaraldehyde, 2.5% formaldehyde in 0.1 M phosphate buffer pH 7.4). Specimens were then rinsed in 0.1 M PIPES buffer, postfixed in 1% buffered osmium tetroxide (1 h), rinsed in buffer, block stained in 2% aqueous uranyl acetate (20 min), dehydrated in an ethanol series and embedded in Spurr resin (Elektron Technology, Stansted, UK) in the normal way. Gold/silver ultrathin sections were then cut from suitable regions of the tissue and stained with Reynolds lead stain and viewed on a Hitachi H7000 transmission electron microscope equipped with a SIS Megaview III digital camera.

Polarized light microscopy

For polarized light visualization, fresh pulp vesicles were carefully extracted and placed into a glass slide, cut by the middle with a scalpel and covered with a glass-cover without any fixative. Images were taken with a Nikon Eclipse 90i (Japan) coupled with a polarization filter.

RESULTS

Changes in colour and pigment composition in peel of oranges and grapefruits at two ripening stages

Changes in peel colour, chlorophyll and carotenoid content were examined in the peel of the ordinary (Navel, N) and two mutants (the yellow Pinalate, P and the red Cara Cara, C) oranges and in white (Marsh, MSH) and red (Star Ruby, SR) grapefruits, at two maturation stages, breaker and full colour. These stages were selected because previous studies have demonstrated that at colour-break occurs the transition from chloroplast to chromoplast, triggering a massive increase in

coloured carotenoids that finally determine the characteristic colouration of full mature fruits (Gross 1987; Kato et al. 2004; Rodrigo et al. 2004; Alquézar et al. 2013). Therefore, we rationalized that both stages are the most appropriated to analyse how the changes in pigment composition could be related to plastids differentiation in peel and pulp tissues.

Peel colour was similar in fruits of three orange varieties at the breaker stage (Table 1). In full coloured fruit, P presented the characteristic pale-yellow colouration and a lower *a/b* ratio (0.16) than N or C, that were similar to each other (around 0.78) (Table 1). On the other hand, grapefruits showed as expected, marked differences in colouration since at that the same ripening stage, white grapefruit was always less coloured than the red SR (Table 1). Chlorophyll content in the peel of P fruit (154 µg g^{-1} FW) at the breaker stage was around 30% higher than in N and C (106 and 100 µg g^{-1} FW, respectively), in agreement with the slow rate of peel colouration in this mutant (Rodrigo et al. 2003). In the peel of grapefruit at breaker stage, chlorophylls were 30% higher in SR (179 µg g^{-1} FW) than in MSH (125 µg g^{-1} FW). In the peel of full coloured fruit of all varieties, only residual amounts (below 5.0 µg g^{-1} FW) of chlorophylls were detected. Analysis of carotenoid profile by HPLC-PDA revealed important qualitative and quantitative differences in the peel of the five varieties. At breaker stage, the peel of P accumulated 3 times (52.4 µg g^{-1} FW) more carotenoids than the parental N (16.3 µg g^{-1} FW) and also the red mutant C (13.9 µg g^{-1} FW) (Fig. 1a). In the peel of mature fruit, total carotenoids increased 7.5-, 9- and 13-times in P, N and C fruits, respectively, compared to values at breaker stage, and the differences between varieties were also maintained (Fig. 1c). Total carotenoids concentration in the peel of MSH grapefruit (8.0 µg g^{-1} FW) was relatively low and similar at breaker and full colour stages, by contrast, SR peel accumulated larger amounts of carotenoids that moderately increased from breaker (41.6 µg g^{-1} FW) to full coloured fruit (55.4 µg g^{-1} FW) (Fig. 1b,d).

Table 1. Changes in peel and pulp colour (a/b Hunter ratio) of fruits of three orange (Citrus sinensis L) varieties (the ordinary orange-coloured N, and the yellow P and the red C mutants) and two grapefruit (Citrus paradisi) varieties (the white MSH and the red SR) at breaker and full colour stages. Data are means ± S.E. of three replicates of ten fruits

	Peel		**Pulp**	
Oranges	Breaker	Full Colour	Breaker	Full Colour
Navel	-0.62±0.02	0.79±0.03	-0.20±0.01	0.02±0.01
Pinalate	-0.66±0.02	0.16±0.03	-0.25±0.01	-0.11±0.01
Cara Cara	-0.60±0.01	0.78±0.02	0.48±0.05	0.54±0.03
Grapefruits				
Marsh	-0.67±0.01	0.07±0.02	-0.30±0.01	0.24±0.01
Star Ruby	-0.29±0.02	0.21±0.05	0.30±0.01	0.54±0.03

As expected, there were remarkable differences in fruit peel carotenoids composition among the varieties studied. The peel of P fruit accumulated substantial amounts of linear carotenoids, mainly phytoene (28.6 µg g^{-1} FW), phytofluene (4.69 µg g^{-1} FW) and ζ-carotene (4.62 µg g^{-1} FW), all of them accounting for 72% of total carotenoids (Fig. 1a). By contrast, phytoene represented only 10% of total carotenoids in peel of N fruits and 36% in the C mutant. At this

breaker stage, other chloroplastic-type carotenoids (i.e. lutein, α- and β-carotene) were also detected in the peel of all orange varieties (Fig. 1a). The increment in total carotenoids during peel colouration was accompanied by a change in their profile (Fig. 1c). Thus, the peel of P mature fruit showed an extraordinary increment in the content of linear carotenoids, mainly in phytoene (263 μg g^{-1} FW) but also in phytofluene (29.5 μg g^{-1} FW) and an important amount of a mix of ζ-carotene isomers (42.2 μg g^{-1} FW), all accounting for 86% of total carotenoids, and the remaining 14% corresponded almost exclusively to 9-(*Z*)-violaxanthin (Fig. 1c). By contrast, the xanthophyll 9-(*Z*)-violaxanthin was predominant in the peel of N and C, accounting for 68 and 57% of total carotenoids, respectively (Fig. 1c). The C_{30} apocarotenoid β-citraurin accumulated progressively with peel colouration of N (2.76 μg g^{-1} FW) and C (4.15 μg g^{-1} FW) oranges but it was not detected in the yellow P mutant (Fig. 1c).

Total carotenoids in the peel of SR grapefruit at both ripening stages were 4-5 times higher than in MSH. In both grapefruits at the breaker stage chloroplastic-type carotenoids represented around 30% of the total content (Fig. 1b). In SR, linear carotenes represented about 40 and 89% of total carotenoids in breaker and full coloured fruit, respectively, with the striking feature of the accumulation of red carotene lycopene (1.6 μg g^{-1} FW) at full coloured stage. By contrast, the peel of mature MSH grapefruit only accumulated minor amounts of carotenoids, mainly phytoene (3.3 μg g^{-1} FW) and violaxanthin (4.98 μg g^{-1} FW; Fig. 1d).

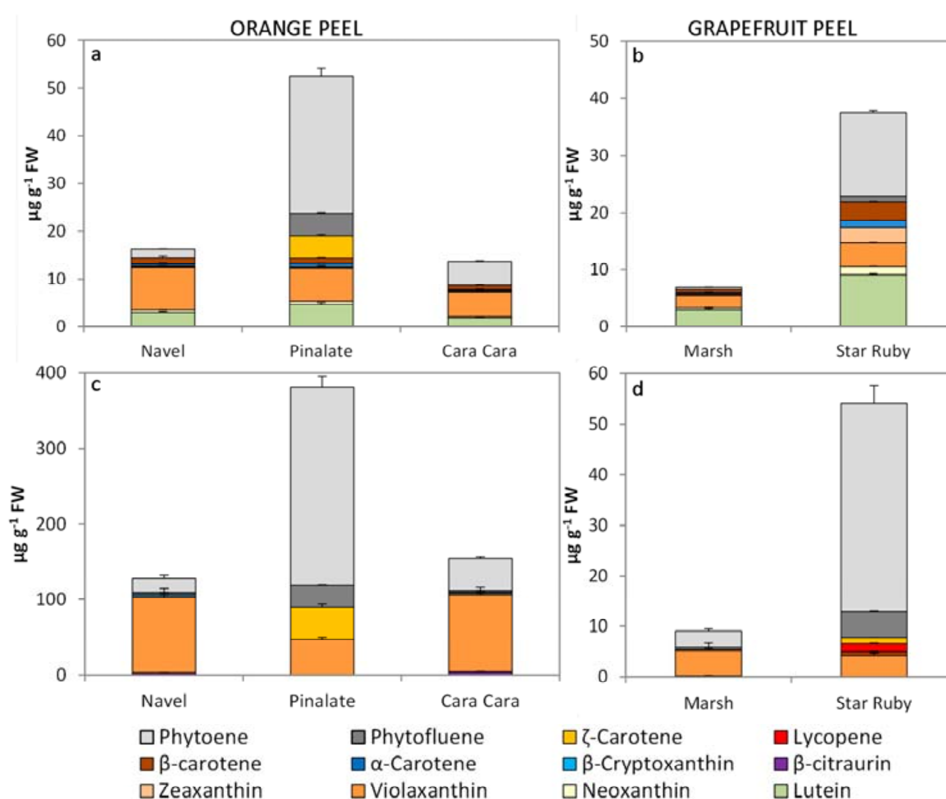

Figure 1. Carotenoid content and composition (μg g^{-1} FW) in the peel of fruit of three orange (*Citrus sinensis* L) (a, c), the ordinary orange-coloured N, and the yellow P and the red C mutants, and two grapefruit (*Citrus paradisi*) (b, d), the white MSH and the red SR, at breaker (a, b) and full colour (b, d) stages. Data of carotenoid contents are means ± SD of three replicates.

Plastid ultrastructure in the peel of oranges and grapefruits at two ripening stages

Thin cut sections of flavedo tissue of the three orange and two grapefruit varieties at breaker and full-colour stage were obtained and the first 4-5 layers of cells underneath the epidermis (Supplementary Fig. S1) were inspected by transmission electron microscopy (TEM) for the presence of chloro- and chromoplasts. In the peel of breaker oranges, chloroplasts were observed surrounding the vacuole of epidermal and outer exocarp cells. Inside the chloroplast, grana composed by stacked thylakoids and starch grains were detected. At this ripening stage, most chloroplast showed an advanced disintegration of thylakoid membranes concomitantly with the development of even round osmiophilic plastoglobuli (Fig. 2a-i). This particular chloroplastic structure was detected in the epidermis and cells of the flavedo of the three oranges varieties and the number of chloroplasts was also comparable with an average of 4.5 per cell. Similarly to oranges, no obvious differences were detected in the chloroplasts structure of both grapefruit varieties at breaker stage, showing similar plastid number per cell as well as a combination of intact thylakoidal membranes with membranes progressively disassembled (Fig. 3a-f). In grapefruit flavedo plastids plastoglobuli were also present, while small starch grains remnants were only detected in MSH (Fig. 3c).

Figure 2. TEM pictures from the chloro- and chromoplasts ultrastructure of the peel of orange fruit (N, P, C) at breaker (a-i) and full colour (j-r) stages. References: (LG) lipid grains, (m) mitochondria, (mm) achlorophyllous membranes, (n) nucleus, (Pg) plastoglobuli, (s) starch grains, (t) thylakoids.

As peel colouration progressed, chloroplasts disappeared and were gradually transformed into chromoplasts. The chromoplasts number was reduced to an average of 2.3 per cell in the epidermis and exocarp of N and C whereas in the orange P mutant the amount of chromoplasts was slightly higher (3.0/cell). Moreover, in P mutant massive amounts of starch grains were easily distinguishable by toluidine blue staining by optical thin cuts micrographs while in ordinary N orange and the red C mutant only low amounts of starch grains could be observed (Supplementary Fig. S1). Besides these particular features (higher plastid density and starch grains) in the peel of P mutant, it was remarkable the massive uneven storage lipid grains (LG) (300-800 nm in diameter) inside the plastids, being less electron-dense than plastoglobuli. These structures appeared to be extruded from the chromoplasts when reaching a particular size or number, and then accumulated in the cytosol surrounded by membranes (Fig. 2o and Supplementary Fig. S4). This phenomenon was hardly observed in C and N chromoplast, where extruded lipid structures formed massive drop-like complexes in the cytosol (Supplementary Figs. S3 and S5). Typical even, round and highly osmiophilic plastoglobuli were present in both N and C chromoplasts, being of small diameter in N (180-370 nm) than in C (180-550 nm) (Fig. 2j, q), while barely detectable in P peel (Fig. 2m-o). In the peel of the three orange varieties, achrolophyllous membranous structures inside peel chromoplasts where detected, forming parallel organized structures (Fig. 2k, m, r).

Chromoplasts from the flavedo of coloured yellow MSH grapefruit presented typical round even plastoglobuli (50-500 nm in diameter), combined with membranous structures (Fig. 3g-i). In the red SR grapefruit, chromoplast contained irregular and light grey grains, round highly osmiophylic plastoglobuli and arranged membranous structures (Fig. 3j-l). The average number of chromoplast per cell was slightly higher in SR (3.0) than in MSH (2.0).

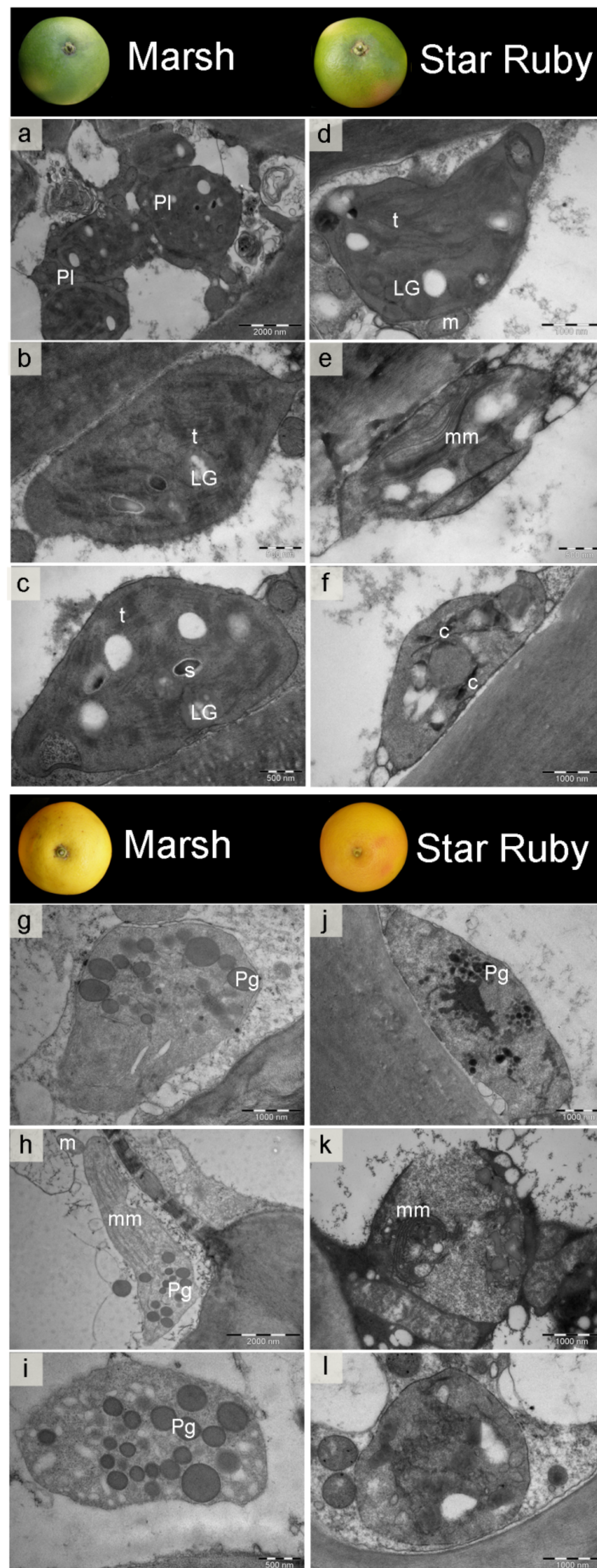

Figure 3. TEM pictures from the chloro- and chromoplasts ultrastructure of the peel of grapefruits (MSH and SR) at breaker (a-f) and full colour (g-l) stages. References: (c) lycopene crystals, (LG) lipid grains, (m) mitochondria, (mm) achlorophyllous membranes, (n) nucleus, (Pg) plastoglobuli, (s) starch grains, (t) thylakoids.

Changes in colour and pigment composition in pulp of oranges and grapefruits at two ripening stages

Changes in colour and carotenoid content and composition were also analysed in pulp of the orange and grapefruit varieties selected at the same ripening stages used for pigment analysis in the peel. The pulp of breaker fruit of N and P oranges was yellowish and similar to each other, but in the C mutant it already displayed a pale red colouration (Table 1, Fig. 5). Differences in pulp colouration were more evident among varieties in mature fruits, where pulp of P registered the lower *a/b* value (-0.11), characteristic of a pale-yellowish colouration, followed by N (0.02) with a slight orange colour and the pulp of C displayed a red tone (0.54) (Table 1). In grapefruits, the pulp of MSH exhibited the typical pale yellow colouration and although increased during repining it remained yellow in mature fruits. By contrast, the red colour of the SR pulp was evident form the breaker stage and increased (from 0.30 to 0.54) with ripening.

Changes in carotenoids content and composition in the pulp of the orange and grapefruit varieties are shown in Fig. 4 and chlorophylls were not detected at any developmental stage. Total carotenoids in the pulp of oranges increased with maturity and were approximately 6-times higher in both mutants compared to the ordinary N orange (Fig. 4a, c). Carotenoid composition was also completely different in the pulp of the two mutants, reflecting their singularities in colouration. In the pulp of the ordinary N orange, β,β-xanthophylls predominated, mainly the *9*-(*Z*) isomer of violaxanthin, which accounted for 55% and 78% of total carotenoids in breaker and full colour stages, respectively. At this later stage, phytoene (1.10 $\mu g\ g^{-1}$ FW), phytofluene (0.19 $\mu g\ g^{-1}$ FW) and the xanthophylls β-cryptoxanthin (0.59 $\mu g\ g^{-1}$ FW) and zeaxanthin (0.21 $\mu g\ g^{-1}$ FW) were also detected (Fig. 4c). In the pulp of both P and C mutants the most striking feature was the extremely high amount of linear carotenes which represented 80-95% of total carotenoids. In P and C pulp 15-(*Z*)-phytoene and phytofluene accounted for 50-80% of the total content, while in P pulp ζ-carotene (mix of isomers) represented between 15 and 19% of total carotenoids, and in C pulp lycopene ranged between 20-14% of the total content of carotenoids. In the pulp of P, the content of β,β-xanthophylls was very low, even at full-coloured stage (2.8 $\mu g\ g^{-1}$ FW), representing only 4% of total carotenoids (Fig. 4c), while in C pulp the concentration of β,β-xanthophylls, mainly *9*-(*Z*) isomer of violaxanthin, was more similar to the parental N, but due to the high concentration of carotenes its percentage over the total was reduced (6.6%; Fig. 4c).

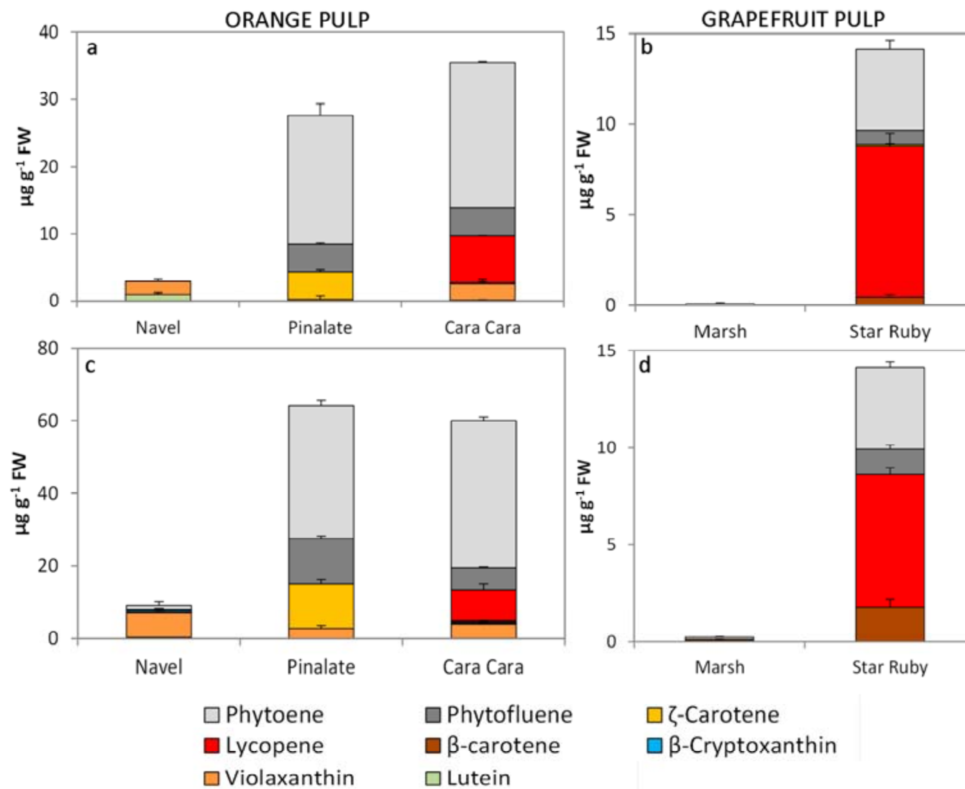

Figure 4. Carotenoid content and composition (µg g^{-1} FW) in the pulp of fruits of three orange (*Citrus sinensis* L) (a, c), the ordinary orange-coloured N, and the yellow P and the red C mutants, and two grapefruit (*Citrus paradisi*) (b, d), the white MSH and the red SR, at breaker (a, b) and full colour (b, d) stages. Data of carotenoid contents are means ± SD of three replicates.

Total carotenoid content in the pulp of the red SR was about 90- and 40-times higher than in the white MSH at breaker and full colour stages, respectively. This latter variety accumulated very low amounts of phytoene (0.12 µg g^{-1} FW), ζ-carotene (0.06 µg g^{-1} FW) and violaxanthin (0.07µg g^{-1} FW) at full colouration. By contrast, SR accumulated linear carotenoids (12.3 µg g^{-1} FW), with major proportion of lycopene and phytoene (50% and 30% of total carotenoids, respectively) and minor amounts of phytofluene and also the cyclic β-carotene (1.80 µg g^{-1} FW) (Fig. 4b, d).

Plastid ultrastructure in the pulp of oranges and grapefruits at two ripening stages

The observation of chromoplasts ultrastructure in cells of the pulp (juice vesicles) from the three oranges and the two grapefruit varieties was also performed, as striking differences in carotenoids content and composition were detected among them. Thin cut sections from vesicle stalks were prepared from individual juice vesicles and cells were inspected for plastids morphology and ultrastructure (Supplementary Fig. S2). At breaker stage of all varieties analysed, no chloroplasts were observed in the pulp cells, however, chromoplasts containing plastoglobuli, membranes and starch grains were detected (Fig. 5a-i). Starch grains were only identified at breaker stage in the pulp of the yellow P and the red C, but not in chromoplasts of the parental N (Fig. 5d, e, g, h). By contrast, arranged membranous structures were observed inside chromoplast of N oranges (Fig. 5a-c) but not in the cells of both C and P mutants. In the pulp of coloured orange

fruit, abundance of plastoglobuli inside plastids increased and the ordinary N orange presented a characteristic type of chromoplast, containing typical round even and highly osmiophilic plastoglobuli of 100-300 nm in diameter (Fig. 5j-l). Interestingly, in the pulp of the yellow P mutant, which accumulated very high amounts of colourless linear carotenoids, two different kinds of chromoplasts were observed. The chromoplast-type I (Fig. 5o) was quite similar to that of N pulp with typical plastoglobuli of 50-200 nm in diameter (Fig. 5j, k). The chromoplast type II of P was the most abundant and presented round and white-coloured (not electron-dense maybe due to sample preparation) vesicles or grains of 250-800 nm in diameter (Fig. 5m, n). Type II and type I chromoplasts were found inside the same cells in a 5:1 ratio. In the pulp chromoplasts of the red C mutant, in addition to the typical plastoglobuli of 200-250 nm in diameter, a new structure was also detected: long and needle-shaped dark structures that resemble lycopene crystals and membranous remnants surrounding them (Fig. 5q, r). The presence and size (2-14 µm long) of crystals in the pulp of C were confirmed by polarized light microscopy (Fig. 7b). These structures were not observed in the pulp of any of the other two oranges (Fig. 7c, d), in agreement with the absence of lycopene in the pulp of these varieties (Fig. 4).

Figure 5. TEM pictures from the chromoplasts ultrastructure of the pulp of orange fruit (N, P, C) at breaker (a-i) and full colour (j-r) stages. References: (c) lycopene crystals with membrane remnants inside[1]; (m) mitochondria, (mm) achlorophyllous membranes, (n) nucleus, (Pg) plastoglobuli, (s) starch grains, (t) thylakoids, arrows indicate the exclusive presence of even and round grains or vesicles in P. [1]After osmium fixation the crystalloids are largely dissolved during the dehydration procedure and their expanded envelopes are shrunken into an undulating shape.

Differences in the structure of chromoplasts from the pulp were even more noticeable in grapefruits, as both varieties also showed dramatic differences in carotenoid content and composition. The red pulp of SR presented a high proportion of crystals in the chromoplasts (2 to 14 µm), that were evident since the breaker stage (Fig. 6d-f) and resembled those present in C chromoplast (Fig. 5q, r). Interestingly, TEM observation suggested the presence of a higher number of crystals in SR grapefruits than in C oranges, which was further corroborated by polarized light microscopy (Fig. 7a, b). Moreover, the presence of typical round even plastoglobuli in combination with crystals was also detected in higher number SR pulp at full colour stage (Fig. 6j-l). In the pulp of the white MSH grapefruits, which was almost devoid of carotenoids, a few number of chromoplasts per cell was observed and, moreover, in MSH chromoplasts crystalloid structures were never detected and it was also noticeable the lack of well-defined structures (Fig. 6a-c). As ripening progressed, MSH pulp chromoplasts developed uneven oval structures, most likely for carotenoid accumulation, which were mainly localized surrounding the cell nucleus (Fig. 6g-i).

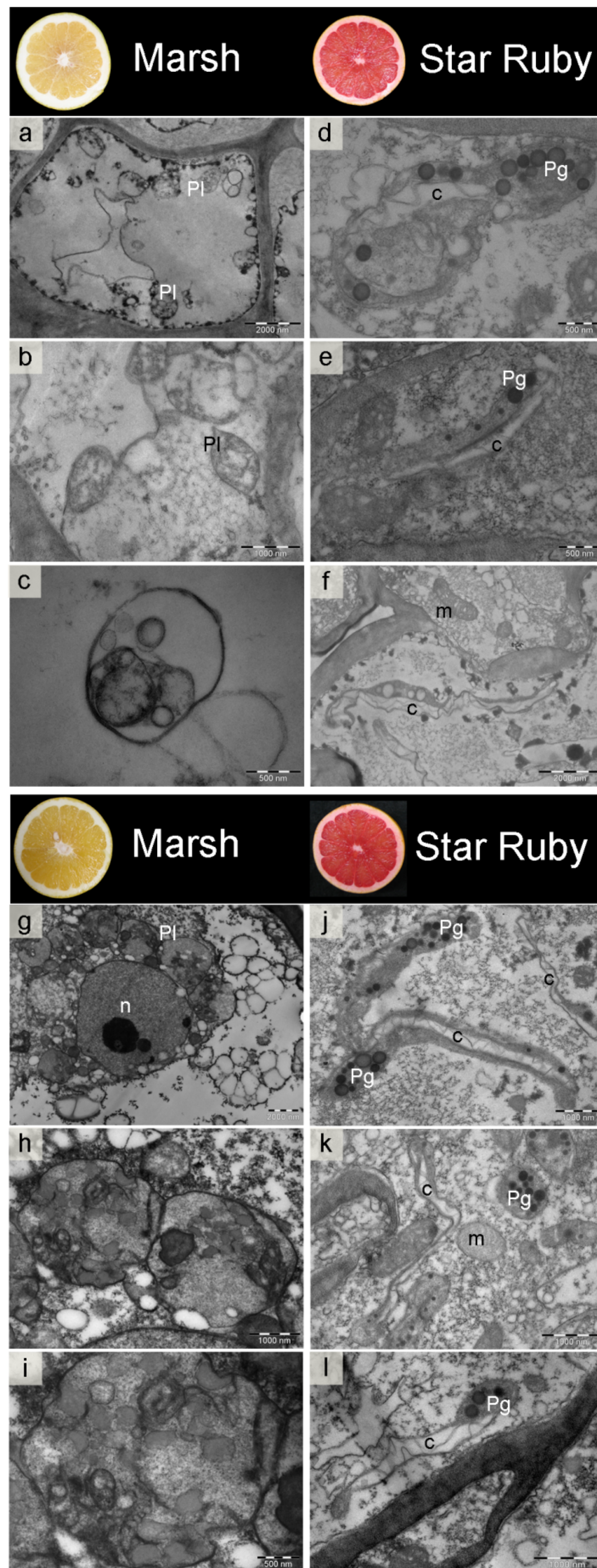

Figure 6. TEM pictures from the chromoplasts ultrastructure of the pulp of grapefruits (MSH and SR) in breaker (a-f) and full colour (g-l) stages. References: (c) lycopene crystals with membrane remnants inside[1], (n) nucleus, (Pg) plastoglobuli, (Pl) plastids. [1]After osmium fixation the crystalloids are largely dissolved during the dehydration procedure and their expanded envelopes are shrunken into an undulating shape.

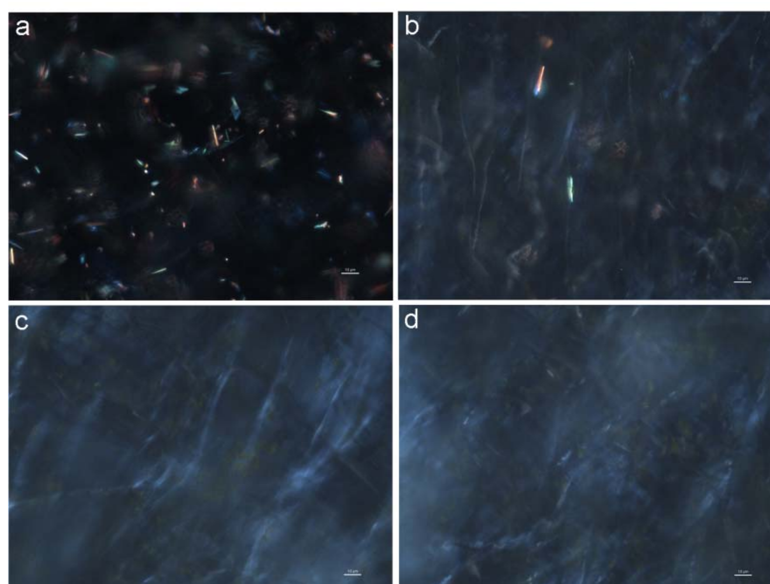

Figure 7. Polarized light microscopy pictures of the pulp of the red SR grapefruit (a), the red C orange mutant (b), the yellow P orange mutant (c) and the ordinary N orange (d) from full-coloured fruits. Note the presence of lycopene crystals only in pulp of red varieties, highly abundant in SR grapefruit (a) and more scarcely in C orange (b).

DISCUSSION

Carotenoids are the main pigments in citrus fruits and exert a deep impact in their organoleptic and commercial quality (Kato 2012; Rodrigo et al. 2013). In addition, human consumption of specific carotenoids is of special relevance due to their dual effect on nutrition and health-related benefits (Berman et al., 2014). Over decades, growers and breeding programs have used the genetic diversity among the *Citrus* genus to select new cultivars and spontaneous and induced mutations differing in either external and internal fruit quality, in which colouration is one of the most remarkable selection parameters (Kato 2012; Garcia-Lor et al. 2013; Rodrigo et al. 2013). Consequently, a great diversity of carotenoids profiles has been created, which constitute an excellent experimental material to explore the biochemical and molecular bases governing citrus fruit pigmentation. Carotenoid accumulation in coloured tissues is tightly linked to its sequestration in chromoplast as specific biosynthetic and sink organelle (Gross 1987; Cazzonelli and Pogson 2010; Li and Yuan 2013). It is well known that in citrus fruit, the more relevant changes at plastidial level occur in the transformation from chloroplast to chromoplast during natural colour change when thylakoids start to disappear and carotenoid-storing structures, known as plastoglobuli, increase in size and number (Thomson 1966; Shimokawa et al. 1978; Gross 1983; Ljubesic 1984; Huyskens 1985; Gross 1987). Nevertheless, scarce information is available about how the development and rearrangement of specific subcellular structures in citrus fruit chromoplasts could be influenced by the contrasting and diverse carotenoid composition existing in this crop; or whether the formation of particular sink structures can determine carotenoid composition in fully mature fruit.

In order to identify potential associations between specific carotenoid profiles and sub-plastidial structures, an in-deep comparative analysis of the ultrastructural changes taking place during chromoplast differentiation was performed in five citrus varieties and mutants with

contrasting differences in both carotenoid content and complement. Moreover, due to the existence of striking differences between peel and pulp carotenoid content in citrus fruit, both tissues were investigated independently. To perform this study an ordinary Navel orange was selected which represents the standard changes occurring in sweet orange chromoplasts. In addition, two spontaneous orange mutants, Pinalate (P), with yellow peel and pulp, and Cara Cara (C), with red pulp, and two grapefruit varieties, white (MSH) and red (SR), were included in this work, since they notably differ in carotenoid complement in both tissues.

In the five varieties considered, carotenoid content was at least three times higher in the peel than in the pulp (Figs. 1 and 4), in agreement with previous reports (Xu et al. 2006; Alquézar et al. 2008; Kato 2012; Alquézar et al. 2013). Moreover, the qualitative composition of carotenoid was also different between both tissues, reinforcing the notion of an independent regulation of carotenoid biogenesis (Alquézar et al. 2008; Matsumoto et al. 2009), which may be also related to the absence of communication or metabolite exchange between peel and pulp in citrus fruit (Tadeo et al. 2008). Therefore, this carotenoid tissue-specific diversity was of special relevance to investigate the changes that occur at the plastidial level that encompass the evolution in carotenoid composition during ripening.

In the peel of ordinary sweet orange, the content of characteristic chloroplastic carotenoids (lutein, α- and β- carotene, zeaxanthin and neoxanthin) is normally reduced during colour change while the synthesis of β,β-xanthophylls is boosted, accumulating mainly 9-(*Z*)-violaxanthin (Kato et al. 2004; Rodrigo et al. 2004; Kato 2012; Rodrigo et al. 2013). According to that, in all varieties analysed, chloroplastic-type carotenoids were detected in the fruit peel at breaker but not at full colour stage (Fig. 1). The main plastidial structures observed in the peel at this stage were stacked thylakoids arranged conforming the typical chloroplastic grana and combined with starch grains (Fig. 2a-i, Fig. 3a-f), resembling the plastidial organization described in citrus leaves (Freeman et al. 1978). Only plastids in an advanced stage of transition from chloro- to chromoplasts showed thylakoid membranes disassembly, starch grains disappearance and the gradual development of the different sink structures for accumulation of newly synthetized carotenoids (Fig. 2a, h).

In agreement with previous reports (Rodrigo et al. 2003; Alquézar et al. 2008; Alquézar et al. 2013), some relevant differences in carotenoid composition were already observed among the five varieties in the fruit peel at breaker stage: an unusual accumulation of the upstream carotenes phytoene, phytofluene and ζ-carotene in P, phytoene in C, whereas in SR all carotenoids were at higher concentration than in MSH grapefruit (Fig. 1a, b). However, besides the existence of important differences on carotenoid complement among the varieties analysed (Fig. 1a, b), only minor variations in chloroplast ultrastructure were observed at breaker stage (Fig. 2a-i and Fig. 3a-f). Thus, it appears that P, C and SR chloroplast have somehow the ability to sequester significant amounts of unusual carotenes without greatly affecting plastidial conformation. This fact could be directly related to the presence of dense thylakoidal membranes in these plastids, which have been demonstrated as an active site for carotenoids biosynthesis and storage in other fruit (Nogueira et al. 2013).

A feature observed in orange peel plastids at breaker stage was the presence of starch grains (Fig. 2 a-i). It has been reported that P mutant has 3-4 times higher starch content compared to its parental (Holland et al. 2005) and, starch grains size and number notably increased in the

yellow P mutant as fruit ripened, which was less noticeable in N and C plastids (Supplementary Fig. S1). This alteration in starch accumulation and sugar metabolism in P may be linked to changes at the subplastidial structures and carotenoid composition (Fig. 1c), as has been recently reported in transgenic tomato fruit where a higher content of upstream carotenes was also related to changes in sugars, amino acids and organic acids levels (Nogueira et al. 2013).

In the pulp tissue of all the varieties studied, the ultrastructural transition from breaker to full colour was less noticeable, since no chloroplasts were detected in this tissue and changes mainly consisted in the appearance of new carotenoid-sequestering structures (Cazzonelli and Pogson 2010; Li and Yuan 2013). Therefore, the absence of pre-existing chloroplast in citrus pulp cells suggests a direct differentiation from proplastids, similarly to what occurs in papaya (Schweiggert et al. 2011) and peach palm tropical fruit (Hempel et al., 2014). In contrast to the observations in peel tissue, important differences in plastid ultrastructure among varieties were already detectable in the pulp at breaker stage (Fig. 5a-i and Fig. 6a-f), coinciding with a contrasting carotenoid composition. C and SR pulps accumulated mainly phytoene and the red lycopene while P presented high amounts of linear phytoene, phytofluene and ζ-carotene (Fig. 4a, b). Ordinary sweet N orange accumulated lower amount of carotenoids at this stage compared to both mutants, mainly 9-(*Z*)-violaxanthin and lutein (Fig. 4a) while MSH showed only minute amounts of carotenoids (Fig. 4b). The noticeable differences between peel and pulp tissues at the plastidial ultrastructure at this breaker stage could be related to the scarce presence of membranes in the plastids from the juice vesicle cells, and therefore, with an earlier need to develop new deposition sites to sustain carotenoid accumulation.

Interestingly, differences in starch accumulation were also detected in the pulp tissue among varieties and the presence of starch grains at breaker stage was restricted to plastids from the orange mutants P and C (Fig. 5d-h), whereas were not observed neither in N orange nor in MSH and SR grapefruit pulps (Fig. 5a-c, Fig. 6a-f). Differences in the pulp chromoplasts at breaker stage were related to the exclusive presence of achlorophyllous membranes in N orange (Fig. 5a-c), since were not detected in any the other four varieties, which disappeared as the fruit ripen (Fig. 5j-l). The reason for this difference remains to be elucidated and could be somehow linked to the lack of starch grains observed in N plastids. Another distinctive feature of C orange and SR grapefruit pulp chromoplasts was the presence of needle-like crystals (Fig. 5i, q, r and Fig. 6 d-f), coinciding with the presence of lycopene in this tissue and resembling those previously described in other lycopene-accumulating fruits (Simkin et al. 2007; Schweiggert et al. 2011; Jeffery et al. 2012; Nogueira et al. 2013) and in the pulp and peel of red grapefruit (Cao et al. 2012; Jeffery et al. 2012; Lado et al. 2015). By contrast, at breaker stage in MSH pulp, the low content of carotenoids was related to a lack of any defined sink structures (Fig. 6a-c).

The notable differences found in the pulp plastids at breaker stage of the five varieties analysed were maximised in ripe fruit, coinciding with major differences in carotenoid composition (Fig. 4c, d). The pulp of full coloured C and P fruit presented at least 6 times more carotenoids than the parental N, with extremely high quantities of the linear phytoene in P and C pulp, a moderate content of phytofluene and ζ-carotene in P, and lycopene in C (Fig. 4c). The pulp of SR grapefruit accumulated 40 times more carotenoids than MSH and the presence of phytoene and lycopene was especially relevant (Fig. 4d). The singular composition of carotenoids in P pulp was associated with

the presence of a novel plastid type composed by non-electrodense, even and round vesicles or globules (Fig. 5m, n). These kind of round even vesicles were similar to that described in an *Arabidopsis* mutant with a disruption in the phytoene desaturase gene leading to the accumulation of high amounts of phytoene (Qin et al. 2007), and also resemble those described in peach palm (*Bactris gasipaes*), accumulating the rare γ-carotene (30% of total carotenoids; Hempel et al. 2014). Therefore, this type of globule seems to be related to the massive accumulation of linear carotenoids in P juice vesicle cells. Interestingly, these even round globules abundant in P pulp were not observed in any of the peel chromoplasts of the three orange varieties studied, instead, huge uneven lipid grains (LG) were detected (Fig. 2m-o), but the composition of these LG remains to be determined. These LG were organized inside the chromoplasts or even extruded from the plastids to the cytosol, probably when reaching certain size or abundance thresholds (Fig. 2o, Supplementary Fig. S4). It has been recently suggested that compartmentalization of carotenoids could be a cellular mechanism to remove the excess of carotenoids accumulated in membranes or inside plastids to alleviate potential cell damaging effects in carotenoid-rich tissues (Nogueira et al. 2013). The presence of similar lipid bodies in the cell cytoplasm has been previously described in chili pepper (Liu 2013) and in avocado fruit (Platt-Aloia and Thompson 1981), where they may coalesce and form few larger lipid bodies in the cytoplasm similarly to that observed in oranges. LG structures were observed in the sections prepared from the peel of the three oranges studied (Supplementary Figs. S3 and S5), however, they were more abundant in P mutant and hardly observed in N orange, coinciding with the differences in carotenoid content and composition observed between these varieties (Fig. 1c). Moreover, these sub-structures never were found in MSH and SR grapefruit (Fig. 6), which showed a reduced total carotenoid content in this tissue compared to oranges.

Plastoglobuli were present in pulp chromoplasts of all varieties at this stage, with the exception of MSH grapefruit (Fig. 5a-i, Fig. 6a-f), and constitute a key site for carotenoid biosynthesis and deposition in plants, being a common structure present in coloured carotenoid-accumulating tissues (Ytterberg et al. 2006; Bréhélin et al 2008; Li and Yuan 2013). Plastoglobuli are highly linked to membranes, since they were proposed to be developed attached to thylakoids and other plastidial membranes and afterwards released into the stroma (Ytterberg et al. 2006; Bréhélin et al. 2008). Plastoglobuli represent an excellent carotenoid-sequestering sink with at least two objectives: to alleviate potential damaging effects from carotenoid accumulation in membranes and to avoid further desaturation reactions as a way of regulating flux of the pathway by partitioning carotenoid precursors from their biosynthetic enzymes (Nogueira et al. 2013). Interestingly, typical plastoglobuli were not found in P mutant peel chromoplasts (Fig. 2m-o), but were otherwise abundant in C and N (Fig. 2j-l, q). It has been proposed that plastoglobuli constitute the main site for xanthophylls accumulation in oranges (Thompson 1966; Gross 1987; Zeng et al. 2011) and the presence of plastoglobuli has been also reported in other citrus such as pummelo (Gross et al. 1983), kumquat (Huyskens et al. 1985), Valencia oranges (Thomson 1966), Satsuma mandarins (Shimokawa et al. 1978) and lemon (Ljubesic 1984). Our results are in agreement with these observations, since both N and C peel accumulated high content of xanthophylls (more than 70% of total carotenoid content), while they only represented 10% of total carotenoids in fully ripen P (Fig. 1c). Apart from xanthophylls, these plastoglobuli have been recently demonstrated to

accumulate phytoene in tomato (Nogueira et al. 2013). In this sense, it is worth noting that in MSH grapefruit peel, with low carotenoid content but nearly 80% being phytoene and xanthophylls, and in SR, rich in phytoene (30%), the predominant sub-chromoplast structures also were plastoglobuli (Fig. 3g-i, Fig. 6j-l). Therefore, plastoglobuli could represent an important sink for phytoene and xanthophylls accumulation in ordinary citrus fruit. Moreover, it is well known that main xanthophylls in ripen citrus fruits are esterified with fatty acids conferring a greater compound stability (Gross, 1987) and recently, it has been demonstrated that plastoglobuli are a main site for accumulation of esterified xanthophylls in chromoplastic tissues (Ariizumi et al. 2014). The presence of achlorophyllous membranes in the full coloured flavedo chromoplasts of all varieties could be related to the need for an effective anchor site for proteins complexes where carotenoid biosynthesis eventually takes place, as has been reported in tomato (Nogueira et al. 2013) and pepper (Deruére et al. 1994). Similar membranes were described in the chromoplast from pummelo (Gross 1983). Chemical content and composition of such membranes have been investigated, containing mainly lipids (mostly galactolipids), carotenoids (like phytoene or β-carotene) and certain proteins such as fibrillins or plastoglobulins, as well as key carotenoid biosynthetic enzymes (Gross 1987; Nogueira et al. 2013). Therefore, membranes could represent a relevant structure necessary for carotenoid biosynthesis in the chromoplasts of citrus fruit peel, since their presence was usually related to a boost in carotenoid content.

Accumulation of (*all-E*)-lycopene in the pulp of C orange mutant and SR grapefruit was linked to the detection of abundant needle-shaped crystalloid structures, surrounded by membrane remnants due to fixation and sample preparation (Fig. 5q, r and Fig. 6j-l), that were confirmed by polarized light microscopy and varied in size from 2 to 14 μm (Fig. 7a, b). These crystals could allow lycopene accumulation and limit isomerization reactions, as has been proposed for (*all-E*)-isomers of β-carotene in carrot roots (Kim et al. 2010), mango (Vasquez-Caicedo et al. 2006; Jeffery et al. 2012) and (*all-E*)-lycopene in tomato (Simkin et al. 2007, Nogueira et al. 2013). Carotenoid-containing crystals development was firstly described to occur inside the lumina of some thylakoids, exhibiting an external membrane even in mature chromoplasts (Ljubesic et al. 1991). However, more recently, crystalloid structures have been described to involve an invagination from the inner envelope of the plastid, indicating that the newly synthetized membranes are not dependent on pre-existing thylakoids (Simkin et al. 2007). Therefore, the presence of a previous thylakoidal membrane is not a prerequisite for crystal formation in citrus fruit, although the existence of surrounding membranes was always linked to lycopene crystals in SR and C pulp (Figs. 5q, r and 6j, l). It is worth noting that despite the similar lycopene concentration in the pulp of C mutant and SR grapefruit (Fig. 4c, d), TEM micrographs and visualization of juice vesicle membranes by polarized light microscopy revealed a higher crystal abundance in SR pulp (Fig. 7a, b). The reason for this remains to be elucidated but could be related to possible differences in the mechanisms that lead to lycopene accumulation in both varieties (Alquézar et al. 2008; Alquézar et al. 2013) and to the relative importance of lycopene in relation to other carotenoids, accounting for 48% in the pulp of full coloured Star Ruby and for 20% in C orange mutant (Fig. 4c, d). Moreover, it has been recently demonstrated that in peach palm fruit, β-carotene, lycopene and γ-carotene occur predominantly in lipid-dissolved and not in crystalline form (Hempel et al. 2014). Therefore, it is tempting to speculate that in C pulp lycopene may be

partially deposited as lipid-dissolved structures influenced by the presence of other `accompanying-carotenoids´, mostly xanthophylls.

According to the carotenoid differences between the citrus varieties considered in this study, chromoplast ultrastructure in the flavedo and pulp tissues was strongly influenced by both, the quantity and the presence of certain individual carotenoids. However, this effect was also influenced by the presence or absence of thylakoidal membranes. At full colour stages, plastoglobuli were the more representative substructures found in citrus chromoplasts, both in the peel and the pulp, with the exception of P peel chromoplast. Results showed that differences in carotenoid composition among citrus varieties conducted to the development of diverse sub-chromoplast structures for synthetizing, accumulating and assuring chemical stability of certain carotenoids. In this sense, plastoglobuli, lipid grains and achlorophyllous membranes were the main structures for carotenoid accumulation in mature citrus fruit, while (*all-E*)-lycopene was preferably stored inside crystalline structures in the red pulp of SR grapefruit and C sweet orange. The former allows the classification of citrus chromoplast into a globular-membranous type while the presence of certain carotenoids (such as lycopene), conduct to the development of crystalloid chromoplast. Similarly, alterations in carotenoid complement found in the citrus orange mutant P of pale yellow colouration, lead to the development of a novel sub-chromoplast structure most likely containing high amounts of linear carotenes. In this sense it is worth to mention that to our knowledge the peel of P fruits contains the highest carotenoid concentration reported in a citrus fruit (nearly 400 µg g^{-1} FW) and one of the top in phytoene among fruits (263 µg g^{-1} FW) (Gross, 1987). Therefore, we conclude that citrus plastids have an extraordinary plasticity to rearrange carotenoids inside different compartments to allow massive pigment accumulation, probably as a mechanism to avoid potential harmful effects. Future work should be focused on investigating the molecular and biochemical mechanisms governing these changes in the different citrus varieties.

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SUPPLEMENTARY MATERIAL

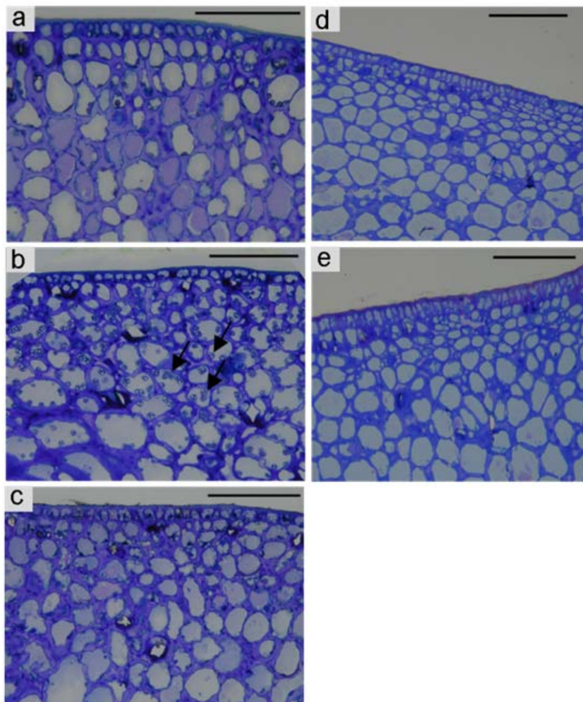

Figure S1. Optical pictures of the thin cuts of the flavedo of (a) Navel, (b) Pinalate, (c) Cara Cara oranges and (d) Marsh and (e) Star Ruby grapefruits stained with toluidine blue. Starch grains inside plastids are pointed by arrows. (Bar-100µm).

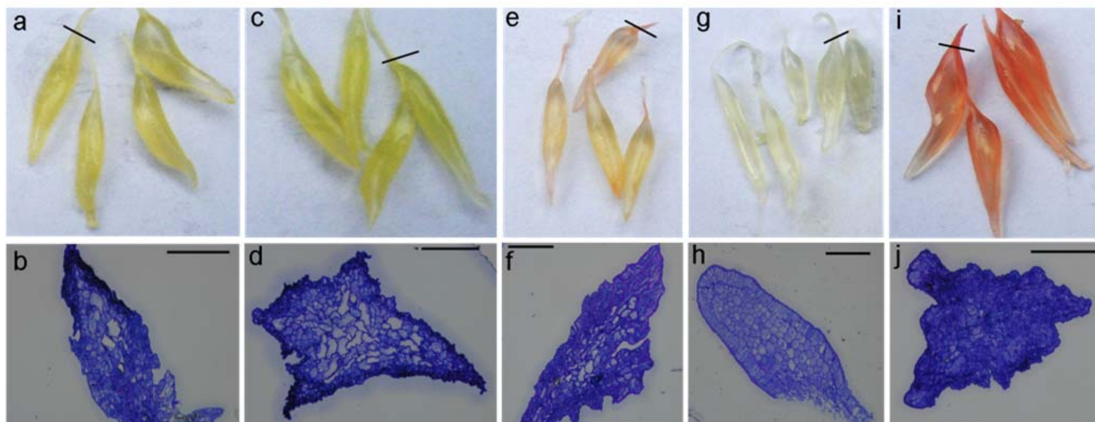

Figure S2. Fruit pulp vesicles and optical micrographs of the thin cuts from (a-b) Navel, (c-d) Pinalate, and (e-f) Cara Cara oranges and (g-h) Marsh and (i-j) Star Ruby grapefruits. Dark lines represent the stalk zone where microscopy cuts were realized (bar- 100µm).

Figure S3. TEM micrographs of Navel orange (a-f) thin cuts from flavedo cells showing lipidic extrusions accumulated in the cytosol (arrows). (cw) cell wall, (Pg) plastoglobules, (Pl) plastids.

Figure S4. TEM micrographs of Pinalate mutant (a-f) thin cuts from flavedo cells showing lipidic extrusions accumulated in the cytosol (arrows). (cw) cell wall, (LG) lipid grains, (Pl) plastids.

Figure S5. TEM micrographs of Cara Cara mutant (a-f) thin cuts from flavedo cells showing lipidic extrusions accumulated in the cytosol (arrows). (cw) cell wall, (LG), lipid grains, (Pg) plastoglobules, (Pl) plastids.

4. DISCUSIÓN

La conservación a bajas temperaturas es posiblemente la tecnología más ampliamente utilizada para preservar la calidad y prolongar la vida útil de frutos y hortalizas durante la postcosecha. Sin embargo, existen muchas especies de origen tropical y subtropical que son sensibles a estas condiciones de conservación y pueden manifestar diferentes fisiopatías, denominadas genéricamente como daño por frío (DF) (Sevillano et al., 2009), entre las que se encuentran los frutos cítricos (Lafuente y Zacarías, 2006).

La sensibilidad al DF en los cítricos varía entre las distintas especies e incluso variedades, así los frutos de pomelos, limones y limas se clasifican dentro de los más sensibles, aunque existen también mandarinas que manifiestan DF, como es el caso de la variedad Fortuna (Schirra et al., 1998; Lafuente et al., 2001; Lafuente y Zacarías, 2006). La alta sensibilidad de los frutos de pomelo a desarrollar DF es un importante problema comercial en los países productores y exportadores de esta fruta, pero también puede suponer un buen modelo experimental para estudiar diferentes procesos y factores relacionados con el DF en estos frutos, lo que ha constituido gran parte del trabajo de esta Tesis Doctoral.

Uno de los resultados más relevantes del presente trabajo ha sido comprobar que la presencia de licopeno en la piel de los frutos de pomelo, tanto en la variedad de piel amarilla Marsh (MSH) como en la de piel roja Star Ruby (SR), evitó de forma importante la aparición de DF. La incidencia de esta alteración no fue homogénea en toda la superficie del fruto, y en el caso específico del pomelo SR, los daños se limitaron exclusivamente a las zonas amarillas de la piel, permaneciendo las zonas rojas prácticamente intactas (Fig. 1, Capítulo 1). En el caso del pomelo MSH, los DF se manifestaron en toda la superficie del fruto, lo que coincidió con una coloración amarilla homogénea en esta variedad (Fig. 1, Capítulo 1). Este resultado es coincidente con observaciones previas realizadas durante años en distintas zonas productoras de cítricos, donde se había señalado una importante variabilidad entre las diferentes zonas de la piel del fruto a desarrollar DF, y donde las partes más coloreadas parecen desarrollar menos síntomas (Grierson, 1986). Además, nuestras observaciones mostraron que incluso en frutos de pomelo SR recolectados y conservados en frío antes de completar la coloración, las zonas verdes de los mismos eran más susceptibles que las rojas a desarrollar daños. Todo ello sugiere una implicación del licopeno en la tolerancia de los frutos de pomelo a los DF.

Las diferencias en la susceptibilidad a los DF entre zonas de distinto color, tanto entre frutos de distintas variedades como entre distintas zonas de un mismo fruto, se confirmaron al visualizar el tejido mediante microscopía electrónica. Se detectaron cambios significativos a nivel celular únicamente en las zonas amarillas de los frutos, mientras que las zonas rojas permanecieron sin modificaciones aparentes (Fig. 2A, Capítulo 1). Los cambios en las células de las zonas amarillas dañadas se caracterizaron por una compresión del citoplasma, formando grandes complejos lipídicos en los que no fue posible detectar orgánulos celulares, combinado con una clara separación de la membrana plasmática de la pared celular (Fig. 2B-C, Capítulo 1), similares a los descritos en otros frutos sensibles al frío (Concellón et al., 2007). También se observó una constricción de las paredes celulares en las células de las zonas del tejido con DF (Fig. 2B-C, Capítulo 1), pero no en las de las zonas rojas (Fig. 2A, Capítulo 1). Estas observaciones son similares a las realizadas anteriormente en frutos de pomelo MSH, con un colapso y degradación del citoplasma y la vacuola en las células epidérmicas y sub-epidérmicas (Platt-Aloia y Thomson,

1976). Estos cambios morfológicos a nivel de la pared celular en el tejido dañado pueden deberse a cambios en la composición de la misma que provocarían un aumento en la permeabilidad que finalmente conlleva a la muerte celular. El mantenimiento de la estructura de la pared celular y de sus componentes (principalmente pectinas y puentes de calcio) se ha asociado a una mayor tolerancia al DF en frutos sensibles previamente acondicionados con altas temperaturas (Holland et al., 2012), reforzando la importancia de la pared celular e integridad de la membrana en la aparición del DF en los frutos cítricos.

Este conjunto de observaciones hicieron plantear la hipótesis de que la composición diferencial en carotenoides, principales pigmentos responsables de la coloración en los frutos cítricos, podría estar relacionada o ser la responsable de la diferencia en la tolerancia a los DF entre las zonas rojas y amarillas de los frutos de pomelo. El análisis de estos pigmentos reveló la presencia del doble de carotenoides totales y 14 veces más licopeno en las zonas rojas que en zonas amarillas de la piel del pomelo SR (Tabla 1, Capítulo 1). Por otro lado y en concordancia con la hipótesis planteada, los frutos de MSH, altamente sensibles al DF en toda la superficie del fruto, mostraron cantidades muy bajas de carotenoides en la piel (Tabla 1, Capítulo 1). Estos resultados, sin embargo, no demuestran que el licopeno sea el responsable de la mayor tolerancia al frío de las zonas rojas de la piel. Para esto es que, teniendo en cuenta estas diferencias y con el objetivo de comprobar si la composición en carotenoides, principalmente el contenido del caroteno rojo licopeno, podría estar relacionada con la mayor tolerancia al DF en las zonas rojas de SR, se planteó la manipulación del contenido de estos pigmentos en frutos de pomelos MSH y SR, mediante dos estrategias. En un primer abordaje se realizó la modificación química del contenido de carotenoides en los frutos de pomelo MSH, mediante la aplicación postcosecha de un inhibidor de la ciclación del licopeno, el (2-(4-clorofeniltio) trietilamina hidrocloruro)-CPTA (Coggins et al., 1970), el cual indujo coloración roja en los frutos de pomelo MSH (Fig. 3A-B, Capítulo 1) y potenció la acumulación de carotenoides (x 7) y principalmente de licopeno (x 32) 4 días después del tratamiento (Tabla 2, Capítulo 1). Estos cambios a nivel de la composición de carotenoides en frutos tratados con CPTA estuvieron asociados a una menor susceptibilidad a desarrollar DF durante el almacenamiento a 2 ºC (Fig. 3C, Capítulo 1). Los frutos tratados desarrollaron síntomas de DF más tardíamente que los controles y su índice de daño fue significativamente menor al finalizar el almacenamiento refrigerado (Fig. 3C, Capítulo 1).

En una segunda aproximación y, utilizando como estrategia experimental la ausencia de luz durante la maduración del fruto de pomelo SR (Capítulo 4), se comprobó la estimulación de la coloración roja de forma uniforme en toda la superficie del fruto, debido principalmente a la acumulación de carotenos (fitoeno y licopeno). Este fenómeno en los frutos de pomelo SR favorecido en ausencia de luz, se fundamenta en observaciones previas a nivel de campo donde es bien conocido que los frutos de esta variedad que se desarrollan en el interior del árbol o cubiertos por el follaje o incluso en contacto con otros frutos, es decir menos expuestos a la radicación luminosa, presentan una coloración roja más intensa y homogénea. Los cambios a nivel bioquímico, molecular y celular que subyacen en este fenómeno han sido objeto de estudio en otro capítulo de esta tesis (Capítulo 4) y se discuten más adelante. Este proceso experimental para promover y uniformizar el cambio de color rojo en los frutos de pomelo SR se comprobó en diferentes plantaciones, localizadas en distintas zonas y expuestas a condiciones climáticas y de

cultivo distintas. Este efecto se ha descrito también por otros autores y se ha indicado que el máximo efecto sobre la pigmentación de los frutos se consigue cuando a los frutos se cubren en el mes de julio, antes del inicio del cambio de color natural (Hwang et al., 2004). Estas observaciones indican la generalidad del proceso y que puede representar un buen sistema experimental para evaluar la implicación del licopeno en diferentes procesos de la fisiología de los frutos de pomelo. Así, en nuestras condiciones experimentales, y en dos localizaciones (Lliria y Moncada-IVIA), los frutos tapados desarrollaron una coloración roja intensa, y acumularon 5 veces más carotenoides que los fruto no tapados, siendo, el licopeno (38-53%) y el fitoeno (38-49%) los más abundantes. En particular, el contenido en licopeno fue más de 70 veces superior al de los frutos expuestos a condiciones normales de fotoperiodo (Tabla 3, Capítulo 1). Los frutos tapados fueron sustancialmente más resistentes a los DF que los no tapados al conservarlos a 2 ºC. Mientras que en los controles no tapados los síntomas iniciales de DF comenzaron a los 21 días, en los tapados se observaron síntomas leves a los 40 días de almacenamiento (Fig. 4, Capítulo 1). La severidad de los DF al final de la conservación y simulación de vida comercial fue notablemente superior en los frutos no tapados, con índices de DF superiores a 2 (en una escala con un máximo de 3), en comparación con valores inferiores a 1 en los tapados, que mantuvieron una calidad externa comercialmente aceptable durante todo el experimento (Fig. 4, Capítulo 1). A pesar de que el DF en los frutos cítricos ha sido ampliamente estudiado y se han evaluado diferentes alternativas para minimizar su incidencia, los resultados de este trabajo son los primeros en los que se describe una inducción de tolerancia a las bajas temperaturas mediante el incremento de un compuesto endógeno que forma parte de la maduración natural de los frutos de pomelo, como es el licopeno. Esos resultados pueden ser la bases para desarrollar nuevas estrategias experimentales o tratamientos comercialmente viables y factibles en los que mediante el incremento de la concentración en licopeno se pueda, por un lado, mejorar la apariencia y la coloración externa de los frutos de pomelo, pero principalmente aumentar la tolerancia a los DF y posibilitar la exposición a bajas temperaturas, bien durante la conservación o por la aplicación de tratamientos cuarentenarios.

Una de las consecuencias fisiológicas de la conservación de frutos a bajas temperaturas y su relación con los DF, es el incremento de la síntesis de etileno. En frutos de diferentes especies se han observado comportamientos opuestos, donde el etileno puede potenciar o reducir los DF, y en consecuencia, la aplicación de un inhibidor de su percepción como el 1-MCP, puede tener efectos beneficiosos o deletéreos en la conservación refrigerada de los frutos (Watkins, 2006). En el caso de los frutos cítricos, se han comprobado efectos discrepantes, ya que mientras que en la mandarina Fortuna la aplicación de etileno protegió frente a los DF y el 1-MCP los estimuló (Lafuente et al., 2001), en otros híbridos de mandarina se produjo el efecto contrario (Salvador et al., 2006; Edagi et al., 2010). La respuesta de los frutos de distintas variedades de pomelo también es particular, ya que se ha observado un incremento en la producción de etileno por las bajas temperaturas, estimulación de los genes de su biosíntesis y un efecto estimulador por la aplicación de 1-MCP (Schirra, 1992; Sapitnitskaya et al., 2006; McCollum y Maul, 2007).

En este trabajo, se almacenaron frutos de pomelos blancos MSH y rojos de SR durante dos campañas sucesivas y se registró la aparición de síntomas de DF durante 8 semanas de almacenamiento a 2 ºC, así como también se evaluó el metabolismo del etileno (producción de la fitohormona, genes de biosíntesis, percepción y repuesta a la hormona). Los resultados muestran

que los primeros síntomas de DF en las zonas sensibles (color amarillo) de los frutos de pomelo se registraron entre los 14 y 20 días de almacenamiento a 2 ºC, pero sin cambios relevantes en la tasa de producción de etileno en comparación con los frutos mantenidos a 12 ºC (Figs. 1 y 2, Capítulo 2). La aplicación de 1-MCP estimuló notablemente la producción de la hormona a partir de los 30 días de almacenamiento, lo que coincide con resultados previos en esta especie (McCollum y Maul, 2007), pero los efectos sobre los DF fueron variables e inconsistentes, por lo que no se pudieron extraer conclusiones definitivas en relación al efecto del 1-MCP en la incidencia de DF. Estos resultados confirman la existencia de un control autoinhibitorio de la producción etileno, similar al descrito en diferentes situaciones para frutos de otras variedades de cítricos (Riov y Yang, 1982; Lafuente et al., 2001; Alós et al., 2014). Sin embargo, la aparición de DF en frutos de pomelos MSH y SR no provocó un aumento en la producción de etileno en frutos dañados, cuya emisión no se diferenció de los frutos almacenados a 12 ºC, que no manifestaron DF (Fig. 2, Capítulo 2), a diferencia de lo descrito en otros trabajos (Schirra, 1992).

Los genes de biosíntesis y percepción del etileno mostraron diferentes respuestas a las bajas temperaturas. Así, aunque la producción de etileno no se incrementó a 2 ºC, sí lo hizo la expresión de los genes de biosíntesis, tanto *ACS1* y *ACS2,* como *ACO*. En los frutos tratados con 1-MCP se produjo una marcada estimulación de la expresión de estos tres genes, lo que demuestra el papel regulador negativo del etileno sobre los genes de su propia síntesis (Fig. 3, Capítulo 2). Destaca la importante estimulación de la producción de etileno al transferir los frutos desde 2 a 20 ºC, para simular la vida comercial, que coincide con un descenso de la expresión de los genes biosintéticos. Estos resultados demuestran que, de forma similar a lo que ocurre en los frutos de la mandarina Fortuna, las bajas temperaturas no permiten una apropiada actividad enzimática y a pesar de estar claramente estimulados los genes de biosíntesis, la producción de etileno es muy baja. Al recuperar una temperatura óptima para la actividad, se estimula la producción de la hormona, posiblemente a expensas de los altos niveles de mRNA que se habían inducido durante el período de frío. La comparación de los niveles de transcritos en los frutos tolerantes y sensibles a los DF, claramente indica que la expresión de los genes de biosíntesis de etileno se estimula por los daños de frío (Fig. 5, Capítulo 1). Esta conclusión es especialmente relevante, ya que hasta la fecha no se habían podido tener evidencias claras que permitieran establecer estas diferencias, lo que se ha facilitado por la comparación entre zonas rojas (tolerantes al DF) y amarillas (sensibles al DF) de la piel de pomelo SR, correspondientes a frutos tapados y no tapados, respectivamente.

Por otro lado, las variaciones en los genes correspondientes a los receptores de etileno del tipo ETR parecen depender de la isoforma evaluada. Así, *ETR1* y *ETR3*, y principalmente este último, se indujeron tanto por las bajas temperaturas como por los daños por frío. Además, los resultados obtenidos al aplicar 1-MCP parecen indicar que la expresión de los receptores se estimula por la acción del etileno. Se ha demostrado también que la transcripción de los *ETR*s se estimula por la aplicación de etileno exógeno en otras especies cítricas y en distintos momentos de desarrollo del fruto (Katz et al., 2004; John-Karuppiah y Burns, 2010; Alós et al., 2014). Un efecto similar se observó en los factores de respuesta a etileno o "ethylene response factors" (*ERF*s), cuya expresión se estimuló por la aplicación de 1-MCP, lo que sugiere un control negativo de la hormona en la expresión de estos genes. Sin embargo, únicamente la expresión de *ERF2* aumentó por las bajas temperaturas hacia el final del almacenamiento (Fig. 5, Capítulo 1), por lo que estos

factores de respuesta no estarían transcripcionalmente regulados por las bajas temperaturas (Fig. 5, Capítulo 1). Ambos factores, de percepción y respuesta al etileno son reguladores negativos de la señalización del etileno, constituyendo pasos clave en la magnitud de la repuesta a la hormona (Agarwal et al., 2012; Wang et al., 2013; Gapper et al., 2013).

El licopeno es un carotenoide lineal con elevada actividad antioxidante debido a la presencia de 11 dobles enlaces conjugados en la molécula, lo que le otorga la capacidad de inactivar especies reactivas del oxígeno, en especial el oxígeno singlete (Krinsky, 1979; Di Mascio et al., 1989; Aizawa et al., 2011). En los frutos cítricos, se ha descrito una relación directa entre el estrés oxidativo provocado por la presencia de especies reactivas del oxígeno (ROS) y la aparición de DF (Sala, 1998; Sala y Lafuente, 2000). El mayor contenido de este caroteno también está asociado a una menor susceptibilidad al DF de frutos de tomate (Whitaker, 1994), mientras que por otro lado, la aparición de daño provoca una reducción en el contenido de licopeno en este fruto (Rugkong et al., 2011). Teniendo en cuenta estas evidencias, la hipótesis que se planteó en este trabajo fue que la tolerancia a los DF en los zonas de los frutos de pomelo con mayor abundancia de este caroteno, podría deberse a un efecto en los sistemas antioxidantes, potenciando una mayor actividad antioxidante en la respuesta de los frutos al estrés oxidativo desencadenado por las bajas temperaturas. Tampoco es posible descartar una contribución de otros carotenoides en esta mayor resistencia a las bajas temperaturas, ya que los frutos tapados también presentaron un mayor contenido de carotenos lineales incoloros, principalmente de fitoeno. En este contexto, frutos del mutante amarillo Pinalate, que acumula grandes cantidades de fitoeno, fitoflueno y ζ-caroteno, son más resistentes al DF que frutos de la naranja parental (Alférez et al., 2005). En este mismo sentido, la mayor presencia de β-caroteno en los frutos resistentes (Tabla 3, Capítulo 1), también podría contribuir a la menor sensibilidad al frío.

Para comprobar la hipótesis anterior, se evaluó la actividad antioxidante total del tejido por diferentes métodos, así como la actividad y la expresión génica de enzimas antioxidantes (CAT, SOD, APX, GR) y la presencia de otros metabolitos con reconocida actividad antioxidante, como el glutatión (GSH) y el ácido ascórbico (AsA). Como indicadores de estrés oxidativo y daño celular, respectivamente, se evaluó también la generación de peróxido de hidrógeno (H_2O_2) y el daño oxidativo (MDA). El contenido de H_2O_2 fue significativamente mayor en etapas tempranas del almacenamiento en los frutos que presentaron DF, al igual que ocurrió en etapas posteriores con el contenido de MDA (Fig. 1, Capítulo 3), lo que indica un aumento del daño oxidativo como resultado de la peroxidación de lípidos en los frutos control, que manifestaron síntomas intensos de DF, frente a los que no los desarrollaron (Tabla 1, Capítulo 3).

La evaluación de la actividad antioxidante total del tejido de los frutos tapados y no tapados, determinada mediante los métodos clásicos (DPPH, ABTS y ORAC), mostró una mayor actividad en frutos sensibles al frío (Tabla 2, Capítulo 3), lo que podría interpretarse como una respuesta a las bajas temperaturas, o los DF, más que con una protección frente a las mismas. Teniendo en cuenta la función del licopeno como antioxidante, se planteó la evaluación de la capacidad del tejido para inactivar el oxígeno singlete (SOAC) (Aizawa et al., 2011), que en promedio fue entre 2 y 3 veces mayor en los frutos tapados, tolerantes al DF, no solo durante todo el almacenamiento sino incluso en el momento de la cosecha (Fig. 2, Capítulo 3). Los resultados sugieren que los frutos en el árbol parecen estar expuestos a estreses ambientales y que la presencia

de licopeno podría ejercer una función antioxidante protectora frente al estrés por frío. Además, estos resultados ponen de manifiesto que el oxígeno singlete parece ser una de las especies reactivas de oxígeno que intervienen en el proceso oxidativo que causa daño celular, y posiblemente, induciendo los síntomas de DF.

Paralelamente, y con el fin de determinar si otros componentes del sistema antioxidante podrían contribuir a esta protección, se evaluó el contenido de glutatión y ácido ascórbico. No se detectaron diferencias en el contenido de glutatión entre frutos sensibles y tolerantes, mientras que los frutos que maduraron expuestos a la luz presentaron entre 1,5 y 2 veces más ácido ascórbico que los frutos tapados (Tabla 3, Capítulo 3). Estos datos coinciden con una mayor incidencia de daño en estos frutos, lo que sugiere una implicación marginal de este antioxidante en la protección frente al DF en pomelos, contrariamente a lo descrito en otros frutos (El Airaj et al., 2013). De forma similar, ni la actividad ni la expresión génica de las principales enzimas que componen el sistema de respuesta al estrés oxidativo, SOD, APX y GR mostraron diferencias entre frutos sensibles y tolerantes al DF (Figs. 3-4, Capítulo 3). Por otro lado, las bajas temperaturas estimularon la expresión de las dos isoformas *GR1* y *GR2*, lo que se tradujo en un leve aumento en esta actividad una vez transferidos los frutos desde 2 ºC a 20 ºC (Fig. 3A, Capítulo 3), lo que demuestra un efecto importante del frío en reprimir la actividad de esta enzima durante el almacenamiento. El almacenamiento refrigerado no afectó a la expresión de los genes *APX1* y *APX2* (Fig. 3B, Capítulo 3).

La enzima CAT registró un incremento de actividad durante el primer día de almacenamiento en frío en ambos tipos de frutos, para ser levemente superior en los frutos tapados durante el resto del almacenamiento (Fig. 4A, Capítulo 3). Esta mayor actividad en frutos tolerantes podría contribuir parcialmente a la mayor resistencia al frío de estos frutos, como se ha sugerido en otras especies cítricas (Sala y Lafuente, 1999; Sala y Lafuente, 2000). Al igual que en el caso de la enzima GR, la expresión de las isoformas *CAT1* y *CAT2* se estimuló por las bajas temperaturas, explicando posiblemente el importante aumento en la actividad de la enzima al simular la vida comercial a 20 ºC (Fig. 4A, Capítulo 3). Estos resultados ponen de manifiesto también un efecto represor del frío sobre la actividad CAT, la cual se estimula notablemente a 20 ºC (Fig. 4A, Capítulo 3). El conjunto de resultados sugiere que la mayor tolerancia al DF de los frutos de SR con mayor contenido de licopeno estaría ligada a un incremento de la capacidad para inactivar el oxígeno singlete, aunque también es posible una contribución parcial de la actividad CAT en la protección frente al estrés oxidativo en estos frutos. Estos resultados sugieren, por lo tanto, una función protectora del licopeno frente al estrés oxidativo generado por las bajas temperaturas en frutos de pomelo.

Por otro lado, la manipulación del contenido de carotenoides en los frutos de pomelo SR se realizó mediante la modificación de las condiciones ambientales durante el desarrollo y maduración de los mismos. Observaciones previas a nivel de campo en España y en Sudáfrica muestran que los frutos de pomelo rojo que se desarrollan en el interior de la copa del árbol presentan una coloración roja más intensa que los frutos del exterior que permanecen expuestos a la luz durante todo el periodo de crecimiento y maduración (Fig. 2, Capítulo 4). Aprovechando esta característica y con el objetivo de inducir una coloración roja intensa en el flavedo del pomelo SR, frutos del exterior del árbol se taparon en etapas tempranas de desarrollo del fruto (julio) antes del inicio del cambio

de color. Los frutos que permanecieron tapados durante gran parte del periodo de desarrollo y maduración mostraron una rápida pérdida de clorofilas que conllevó a una aceleración en el proceso de desverdización (Fig. 3A-B, Capítulo 4). Los frutos tapados registraron también un aumento de 2 a 3 veces en el contenido de carotenoides, ya significativo desde el mes de octubre, y que se mantuvo hasta el final de la maduración (diciembre) (Fig. 3C, Capítulo 4). Esta respuesta a la ausencia de luz se diferencia de lo descrito en otras especies cítricas, en donde los frutos que crecen en el interior del árbol, presentan menor coloración y contenido de carotenoides, así como una mayor sensibilidad a los manchados en la piel (Cronje et al., 2011; 2013; Lado et al., 2013; Rodrigo et al., 2013a). La luz es, en general, un factor que potencia la carotenogénesis, y se ha observado en frutos de tomate (Schofield y Paliyath, 2005; Azari et al., 2010), pimiento (Simkin et al., 2003), en tejidos vegetativos de la planta modelo *Arabidospsis thaliana* (Toledo-Ortiz et al., 2010) y de otras especies vegetales (Pizarro y Stange, 2009), lo que sugiere la existencia de posibles diferencias en los mecanismos que regulan la biosíntesis y acumulación de carotenoides en el pomelo rojo SR respecto a otros frutos cítricos u otras especies vegetales. Es interesante mencionar que en raíces de zanahoria se ha descrito una respuesta similar, ya que la ausencia de luz durante el desarrollo del tejido de reserva es necesaria para inducir la masiva acumulación de β- y α-caroteno (Fuentes et al., 2012; Rodríguez-Concepción y Stange, 2013). También se ha descrito una mayor acumulación de carotenoides en bayas de dos variedades de uva que maduraron en oscuridad (Oliveira et al., 2004), sugiriendo que esta respuesta depende del tipo de fruto, de la especie e incluso del órgano involucrado.

Las diferencias más notables en la composición en carotenoides entre frutos tapados y no tapados se registraron en el contenido de licopeno, que fue entre 30 y 49 veces mayor en el flavedo de los frutos que maduraron en oscuridad (Tabla 1, Capítulo 4). Este aumento en el contenido de licopeno y también otros carotenos lineales como el fitoeno (x 2-3 veces) y fitoflueno (x 5-6 veces), ocurrió conjuntamente con la reducción en el nivel de xantofilas (2-4 veces menor) y del apocarotenoide ABA (1.5-3 veces menor) en frutos tapados (Tablas 1 y 2, Capítulo 4). Estos resultados indican que en oscuridad hay un bloqueo en la ruta de síntesis de carotenoides por debajo del licopeno, ya que hay una acumulación masiva de los carotenos iniciales de la ruta y una disminución de los metabolitos finales (xantofilas y ABA; Fig. 1, Capítulo 4). Estudios previos han demostrado que la acumulación de carotenos lineales en pomelos rojos está relacionada con una menor expresión y actividad de la enzima licopeno ciclasa 2 (*βLCY2*), específica de frutos e inducida durante la maduración en condiciones normales (Alquézar et al., 2009; Alquézar et al., 2013). El perfil de carotenoides en los frutos tapados parecería indicar que este cuello de botella en la ruta se potencia sustancialmente en ausencia de luz.

El análisis de la expresión de los genes de biosíntesis de carotenoides mostró que la luz estimula su transcripción en pomelo rojo SR (Fig. 4, Capítulo 4), de forma similar a lo descrito en otras especies vegetales (Simkin et al., 2003; Pizarro et al., 2009; Toledo-Ortiz et al., 2010). Este efecto fue muy claro en los genes iniciales de la ruta (*PSY*, *PDS* y *ZDS1*) desde octubre, y más tardíamente, a partir del mes de noviembre, también se observó en los genes de la rama β-β-, *βLCY2a*, *βLCY2b* y *βCHX*, encargados de la producción de β-caroteno y xantofilas, respectivamente (Figs. 1 y 4, Capítulo 4). Resultados similares se han descrito en otras especies vegetales, como es el caso de *Arabidopsis*, en donde se ha demostrado una regulación positiva de la

luz sobre la transcripción de la *PSY*, mediada por factores de transcripción conocidos como PIFs (phytochrome interacting factors) (Toledo-Ortiz et al., 2010). Por otro lado, no se observó un efecto de la luz sobre la transcripción de genes de la biosíntesis de precursores de la ruta MEP *(DXS*, *HDR1* y *GGPPS)* (Fig. 5, Capítulo 4) a diferencia de lo observado en tejidos vegetativos de *Arabidopsis* (Botella-Pavía et al., 2004).

Trabajos previos realizados en el laboratorio han demostrado que el pomelo rojo SR presenta una menor actividad de la enzima *βLCY2*, responsable de convertir el licopeno en β-caroteno específicamente en frutos coloreados, y presenta a su vez dos alelos, *a* y *b*, siendo la actividad ciclasa del alelo *b* prácticamente nula (Alquézar et al., 2009). Se ha descrito también que la abundancia del alelo inactivo es mayor en frutos de pomelo que en naranjas y mandarinas (Alquézar et al., 2009) y que estas diferencias podrían ser las responsables de la acumulación de licopeno en esta especie (Alquézar et al., 2009; Mendes et al., 2011; Costa et al., 2012; Alquézar et al., 2013). En este trabajo hemos comprobado que la ausencia de luz reduce de forma significativa la expresión de ambos alelos (Fig. 4, Capítulo 4), lo que haría aún menos eficiente la ciclación de licopeno, y acentuaría del bloqueo de la ruta a este nivel, estimulando la masiva acumulación del caroteno licopeno. Aparentemente, la expresión de los genes tempranos (*PSY, PDS, ZDS*) que existe en condiciones de oscuridad es suficiente para sustentar la acumulación de licopeno. Además, la entrada de precursores a la ruta de síntesis de carotenoides podría verse favorecida en ausencia de luz, ya que en estas condiciones existe una menor competencia con otras rutas con las que se comparten precursores como es el caso de las clorofilas o plastoquinonas (La Rocca et al., 2007; Reinbothe et al., 2010).

En el pomelo rojo SR, al igual que en zanahoria (Rodríguez-Concepción y Stange, 2013), existe una correlación parcial entre la expresión de los genes de biosíntesis de carotenoides y su acumulación. Por tanto, para entender los mecanismos responsables de la acumulación de carotenoides en estas condiciones es importante considerar posibles modificaciones a nivel estructural y, en particular, en los plástidos, que son los orgánulos responsables de su síntesis y acumulación (Cazzonelli y Pogson, 2010; Li y Yuan, 2013). En este sentido, durante la maduración de los cítricos ocurre la transición de los cloroplastos, plástidos presentes en la piel de los frutos verdes, a cromoplastos, desarrollándose estructuras especializadas para la acumulación de carotenoides (Gross, 1987). El estudio de la ultraestructura de los plástidos en frutos tapados y no tapados del pomelo SR permitió observar una marcada aceleración de la diferenciación de los cromoplastos en los frutos tapados, ya visible a partir del mes de octubre (Fig. 7B-C, Capítulo 4). Este desarrollo temprano de estructuras con capacidad de síntesis y acumulación de carotenoides podría potenciar la capacidad sumidero y favorecer una mayor acumulación de estos pigmentos, como se ha descrito en otras especies (revisado en Cazzonelli y Pogson, 2010 y Li y Yuan, 2013). Dentro de los cambios estructurales más relevantes observados en los plástidos de los frutos tapados cabe destacar el desarrollo anticipado de estructuras de biosíntesis y acumulación de estos pigmentos, los plastoglóbulos (Gross, 1987; Bréhélin et al., 2007; Lundquist et al., 2012). También se observó una degradación acelerada de las membranas tilacoidales y la desaparición de los gránulos de almidón presentes en los cloroplastos típicos de frutos verdes (Fig. 7B-C). La presencia de plastoglóbulos ha sido descrita en diferentes especies cítricas (Gross, 1987; Thomson, 1966; Gao et al., 2011; Zeng et al., 2013) y se ha relacionado con una alta capacidad carotenogénica en

otros frutos (Vasquez-Caicedo et al., 2006; Simkin et al., 2007; Schweiggert et al., 2011). La presencia de gran cantidad de licopeno en la piel de los frutos tapados estuvo directamente asociada a la aparición de estructuras cristalinas (Fig. 7E, Capítulo 4), que se han descrito como las mayoritarias para el almacenamiento de *all-trans*-licopeno en tomate y papaya (Simkin et al., 2007; Schweiggert et al., 2011; Nogueira et al., 2013), ya que favorecen su estabilidad y hacen posible su acumulación. Estas estructuras cristalinas no se observaron en los cromoplastos de frutos no tapados, en donde predominaron los pequeños plastoglóbulos en co-existencia con membranas aclorofílicas (Fig. 7D, Capítulo 4).

Los importantes cambios a nivel estructural observados en los plástidos de los frutos tapados estuvieron acompañados por modificaciones en la expresión de genes relacionadas con la formación de subestructuras cromoplásticas y chaperonas. Tal es el caso de las fibrilinas o plastoglobulinas (*FIB1-CitPAP* y *FIB2*) y de las chaperonas tipo sHSPs (small heat shock proteins, *HSP20-4* y *HSP21*), las cuales se han asociado a cambios en la estructura de los plástidos y en su capacidad de acumular carotenoides durante la maduración en distintos frutos (Moriguchi et al., 1998; Neta-Sharir et al., 2005; Simkin et al., 2007), lo que sugiere un posible papel clave de este tipo de proteínas en el desarrollo de estructuras y complejos multienzimáticos que estimulan y permitan la síntesis masiva de carotenoides.

Es interesante mencionar que la expresión de los genes de las chaperonas *HSP20-4* y *HSP21* fue mayor y más temprana en frutos tapados de SR, y el incremento en su expresión génica coincidió con los mayores cambios a nivel de la estructura de cromoplastos, ya visibles a partir del mes de octubre (Figs. 6 y 7B-C, Capítulo 4). Sin embargo, ambos genes también registraron un aumento de expresión en frutos no tapados aunque más retrasada en el tiempo, lo que coincidió con los mayores cambios a nivel estructural en estos frutos (Figs. 6 y 7D, Capítulo 4). Por tanto, la mayor expresión de estos genes parece relacionarse más con los cambios a nivel estructural que con la acumulación de carotenoides específicos. Por otro lado, los cambios en la expresión de las fibrilinas (*FIB1* y *FIB2*) reflejan que estos genes no parecen estar relacionados con los cambios a nivel estructural observados en pomelo rojo (Fig. 6, Capítulo 4). Esto podría ser una diferencia importante entre el pomelo y otras especies cítricas, debido a que estas proteínas son muy abundantes en los cromoplastos de naranja dulce, en donde podrían cumplir otras funciones (Zeng et al., 2013). La expresión del gen *Or* no se modificó sustancialmente durante la maduración de los frutos tapados, mientras que en los frutos control (no tapados) su expresión se incrementó de forma paralela a la maduración (Fig. 6, Capítulo 4). Recientemente se ha descrito que la proteína Or de *Arabidopsis* interacciona directamente con la enzima PSY, favoreciendo su actividad (Zhou et al., 2015) y que la sobreexpresión de este gen provoca un aumento en la acumulación de carotenoides (Bai et al., 2014).

La clara asociación entre la acumulación masiva de carotenoides en los frutos de pomelo SR y el desarrollo temprano de estructuras subcromoplásticas específicas, hizo plantearse la hipótesis de la existencia de una relación directa entre la composición y contenido de carotenoides y la formación de determinadas estructuras para su estabilización en los cromoplastos. En este sentido, y haciendo uso de la gran diversidad en pigmentación y composición de carotenoides que existe en los frutos cítricos (Garcia-Lor et al., 2013; Rodrigo et al., 2013a), se planteó la caracterización de los cambios en la estructura de los plástidos que tiene lugar durante la

maduración, ya que podría estar directamente relacionada con su capacidad para acumular carotenoides específicos (Capítulo 5). Así pues, se llevó a cabo una comparación del proceso de diferenciación de los cromoplastos de una naranja dulce estándar (Navel), de coloración naranja típica y que acumula principalmente violaxantina (80%), β-criptoxantina y apocarotenoides C_{30} (Rodrigo et al., 2004; Rodrigo et al., 2013a) y dos mutantes espontáneos: la naranja de pulpa roja Cara Cara y la amarilla Pinalate. El mutante Cara Cara presenta un perfil de carotenoides similar a Navel en la piel aunque con una mayor contenido en fitoeno, y su principal peculiaridad es la acumulación licopeno, junto con otros carotenos incoloros en la pulpa (Alquezar et al., 2008a), mientras que Pinalate (Rodrigo et al., 2003) acumula elevadas cantidades de carotenos lineales, principalmente fitoeno, fitoflueno y ζ-caroteno tanto en la piel como en la pulpa (Figs. 1 y 4, Capítulo 5). Adicionalmente, también se incluyeron en este estudio el pomelo blanco MSH que acumula mínimas cantidades de carotenoides y el pomelo rojo SR, que en determinadas condiciones (Capítulo 4) es capaz de acumular importantes concentraciones de carotenos, principalmente fitoeno y licopeno (Figs. 1 y 4, Capítulo 5). En general, durante la maduración natural en la piel de los frutos ocurre una reducción en la proporción de carotenoides típicos de tejido cloroplástico (luteína, trans-violaxantina, α- y β-caroteno) en favor de carotenoides coloreados (Fig. 1, Capítulo 5), mientras que los cambios son menos notables en la pulpa, en donde principalmente se observa un aumento en la cantidad de carotenoides pero no hay cambios importantes en la composición cualitativa (Fig. 4, Capítulo 5). A nivel ultrestructural de los plástidos, las diferencias entre las distintas especies y variedades fueron mínimas en tejidos verdes, a pesar de las diferencias importantes en el perfil de carotenoides (Figs. 1, 2a-i y 3a-f, Capítulo 5). En este estadío predominó la existencia de cloroplastos con los típicos tilacoides organizados en granas y la presencia de gránulos de almidón que variaron en abundancia entre las distintas especies evaluadas (Figs. 2a-i y 3a-f, Capítulo 5). En los cloroplastos, la presencia de membranas fue muy elevada, siendo esta subestructura un lugar muy activo de síntesis y acumulación de carotenoides (Nogueira et al., 2013). La acumulación diferencial de carotenoides durante la maduración estuvo acompañada de cambios relevantes a nivel de la estructura de los cromoplastos, directamente relacionados a su vez con el tipo de carotenoide que se acumulaba mayoritariamente. Es así como el licopeno en la pulpa de naranja Cara Cara o en la piel y pulpa de frutos de pomelo rojo SR, se acumuló en forma de estructuras cristalinas (Figs. 5q, r y 6j-l, Capítulo 5), que no fueron detectados en el resto de las variedades evaluadas (Figuras 5j-o y 6g-i, Capítulo 5). La presencia de cristales de licopeno se confirmó por microscopía de luz polarizada, percibiéndose un tamaño de cristales entre 2 y 14 µm, con una mayor presencia en SR que en Cara Cara (Fig.7, Capítulo 5). Estas diferencias podrían estar relacionadas con la importancia relativa de este carotenoide en relación a otros presentes en la pulpa, mayoritariamente xantofilas, ya que representa un 20% en la naranja roja mientras que en SR significa casi un 50% del total de carotenoides (Fig. 4c,d, Capítulo 5). Estructuras cristalinas similares constituyen el principal sitio de acumulación de β-caroteno en zanahoria (Vasquez-Caicedo et al., 2006; Kim et al., 2010; Fuentes et al., 2012). Sin embargo, en otras especies como mango, se demostró la presencia de β-caroteno en plastoglóbulos, aunque en configuración *cis-* (Vasquez-Caicedo et al., 2006), lo que sugiere que el tipo de configuración de la molécula y, posiblemente, la presencia de otros carotenoides, influye directamente en la estructura desarrollada para su acumulación.

Por otro lado, el elevado contenido de fitoeno, fitoflueno y ζ-caroteno en el mutante amarillo Pinalate (tanto en la piel como en la pulpa), estuvo asociado a la aparición de unas nuevas subestructuras globulares en los plástidos (Figs. 2m-o y 5m, n, Capítulo 5). La presencia de glóbulos similares se ha descrito en los cromoplastos de frutos de coloración amarillenta del chontaduro o pejibaye (*Bractis gasipaes*), el cual acumula una elevada concentración de γ-caroteno (Hempel et al., 2014), un caroteno cuya presencia es muy inusual, por lo que estas estructuras globulares parecen estar directamente vinculadas a la presencia de carotenos lineales o monocíclicos. Paralelamente, se observó una re-distribución de cuerpos lipídicos dentro de los plástidos, los cuales son expulsados hacia el citosol, siendo más intenso el fenómeno cuanto mayor era la acumulación de carotenoides (Fig. 2o, Figs. S3-S5 material suplementario, Capítulo 5). Este mutante también presentó grandes cantidades de almidón en la piel de frutos maduros (Fig. S1, material suplementario Capítulo 5), cuyo mayor contenido ya había sido reportado en esta variedad (Holland et al., 2005), y que podría estar relacionado con los cambios en la biosíntesis de carotenoides como se ha descrito en otros frutos cítricos (Cao et al., 2015) y en frutos tomate (Nogueira et al., 2013). Los cromoplastos presentes en la piel de naranjas y en menor medida, también en pomelos, presentaron membranas aclorofílicas bien organizadas (Figs. 2k, m, r y 3h, k, Capítulo 5), las cuales podrían estar sustentando la acumulación elevada de carotenoides en este tejido en comparación con la pulpa, en donde la presencia de estas estructuras es mucho menor (Fig. 4, Capítulo 5). Por lo tanto, el perfil de carotenoides y, principalmente, la conformación geométrica (isomería *cis-trans*) del carotenoide/es mayoritario/s influye directamente en la estructura en la que se acumulan en los cromoplastos de frutos cítricos. Los resultados demuestran que los cromoplastos de los frutos cítricos presentan una gran plasticidad en el desarrollo de nuevas y diversas subestructuras cromoplásticas, lo que permite una redistribución de estos compuestos en diferentes compartimentos celulares, facilitando su acumulación y estabilización.

5. CONCLUSIONES

1) Las zonas rojas de los frutos de pomelo de la variedad Star Ruby (SR) fueron más resistentes a los daños por frío (DF) y no desarrollaron daño a nivel celular durante la conservación a 2 ºC, respecto a las zonas amarillas del mismo fruto y de los frutos de la variedad Marsh (MSH).

2) Las zonas rojas del pomelo SR tolerantes a los DF tenían mayores niveles de carotenoides totales y especialmente de licopeno. El aumento en el contenido de licopeno en frutos de MSH y SR mediante manipulación química (x32) y ambiental (x75), respectivamente, incrementó notablemente la tolerancia al DF, incluso después de 2 meses a 2 ºC. Estos resultados sugieren que el licopeno puede ser el responsable de la protección frente al estrés provocado por las bajas temperaturas en los frutos de pomelo.

3) Durante la conservación de los frutos de pomelo a 2 ºC no se produjo un aumento significativo de la tasa de producción de etileno, pero sí en la acumulación de trascritos correspondientes a los genes de biosíntesis *ACS1, ACS2* y *ACO,* únicamente en las zonas sensibles a los DF, y no en las resistentes. Estos genes pueden ser, por tanto, buenos marcadores de la sensibilidad de los frutos de pomelo a los DF.

4) La expresión de los genes de los receptores del etileno, *ETR1* y *ETR3*, parece estar relacionada con la incidencia del DF, así como también con la respuesta al frío. El etileno, por tanto, parece regular en forma negativa la expresión de los genes de respuesta a etileno *ERF1* y *ERF2*, durante el almacenamiento en frío y posterior vida comercial a 20 ºC.

5) La inhibición de la percepción del etileno mediante la aplicación del antagonista 1-MCP, indujo notablemente la expresión de los genes biosintéticos (*ACS* y *ACO*), así como la producción de la hormona durante el almacenamiento en frío y al transferir los frutos a 20 ºC, demostrando una regulación auto-inhibitoria de la producción de etileno. No se observó un efecto concluyente de la aplicación de este inhibidor en el desarrollo del DF en los frutos de pomelos.

6) La mayor tolerancia al DF en los frutos de pomelo con un elevado contenido de licopeno se relacionó directamente con una mayor capacidad para inactivar el oxígeno singlete (SOAC). La mayor actividad antioxidante total de los frutos más sensibles a los DF (expuestos a la luz) y su aumento durante la conservación en frío refleja una respuesta a estas condiciones de estrés, no relacionada con la resistencia al DF.

7) La tolerancia de los frutos de pomelo SR al DF no se relacionó con el contenido de ácido ascórbico ni de glutatión en la piel, así como tampoco con la actividad o expresión génica de las enzimas GR, APX y SOD, sugiriendo que los cambios durante el almacenamiento están más bien relacionados con la respuesta al estrés por bajas temperaturas. Sin embargo,

la mayor actividad CAT en los frutos tolerantes (tapados) podría contribuir parcialmente a la menor incidencia del desorden en estos frutos.

8) La ausencia de luz durante la maduración de los frutos de pomelo SR acelera la desverdización, y estimula la coloración roja de la piel, incrementando significativamente la acumulación de carotenos, principalmente de licopeno, y reduciendo la de metabolitos posteriores en la ruta (xantofilas y ABA). Estos cambios están acompañados por una reducción en la expresión de los genes de la biosíntesis de carotenoides, lo que probablemente acentúa el bloqueo existente de la ruta a nivel de la *βLCY2*, promoviendo así la acumulación de licopeno.

9) En las células del flavedo de los frutos de pomelo SR madurados en oscuridad se observa una transición temprana de los cloroplastos en cromoplastos, favoreciendo la degradación de tilacoides y gránulos de almidón, así como la formación de nuevos sub-orgánulos y estructuras (plastoglóbulos y cristales). Estas estructuras podrían facilitar la capacidad sumidero y ayudar a la elevada acumulación de carotenos en estos frutos.

10) Los cambios a nivel ultraestructural que tienen lugar en los plástidos de los frutos de pomelo SR, con elevado contenido en carotenos, estuvieron acompañados por un aumento en la expresión de los genes de las chaperonas *HSP20-4* y *HSP21*, lo que sugiere que pueden contribuir a la síntesis de carotenoides en frutos cítricos.

11) La composición distintiva de carotenoides en la piel y la pulpa de variedades y especies de frutos cítricos con distinta pigmentación está relacionada con el desarrollo de estructuras específicas en los plástidos. Los plastoglóbulos y las membranas aclorofílicas son las estructuras más abundantes en los cromoplastos de la piel de los frutos de naranjas y pomelos, siendo probablemente lugares activos de síntesis y acumulación de fitoeno y β,β-xantofilas. La presencia de licopeno está asociada al desarrollo estructuras cristalinas, mientras que la inusual y elevada concentración de carotenos incoloros en la pulpa del mutante Pinalate dio lugar de forma exclusiva a un nuevo tipo de plástido con grandes subestructuras globulares. Estos resultados muestran la extraordinaria plasticidad de estos orgánulos en los frutos cítricos que permite una redistribución de los carotenoides en diferentes subestructuras que, posiblemente favorecen su acumulación durante la maduración.

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7. ANEXO

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Involvement of lycopene in the induction of tolerance to chilling injury in grapefruit

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Carotenoid

ABSTRACT

Grapefruit are among the more sensitive *Citrus* varieties likely to develop chilling injury (CI) symptoms during postharvest storage at low temperatures. Comparative observations of the incidence of CI in fruit of white Marsh (MSH) and red Star Ruby (SR) grapefruit during postharvest storage at 2 °C plus 7 days at 20 °C to simulate shelf-life revealed that (1) the former was consistently more sensitive to CI, developing cold damage uniformly throughout the whole rind surface, and (2) more strikingly, CI symptoms in fruit of SR grapefruit were restricted to the yellow areas of the rind and the red-colored zones were almost absent of cold damage. This tolerance to CI in red flavedo was associated with high carotenoid (×2) and lycopene (×14) contents, as compared with yellow-colored flavedo. Absence of chilling damage in red areas of SR grapefruit rind was confirmed by cellular ultrastructure observations, in which these epidermal cells were intact, with a well-defined structure and compact vacuoles filled with content. Cells of yellow-colored tissue developing CI, were collapsed, with a contracted vacuole and shrinking organelles. To explore whether the tolerance to CI in red areas of grapefruit rind was due to an elevated lycopene concentration, chemical and environmental stimulation of this carotenoid was performed in fruit of both grapefruit varieties. Application of the inhibitor of the lycopene cyclase activity, CPTA (2-(4-chlorophenylthio) triethylamine hydrochloride) induced red coloration, increased lycopene accumulation (×32) and significantly delayed development of CI symptoms in the rind the CI-sensitive MSH. Bagging of SR grapefruit enhanced a homogenous red coloration and substantially induced lycopene accumulation (×75). CI symptoms in bagged fruit were notably delayed and reduced, as compared with non-bagged yellow fruit, upon subsequent storage at 2 °C for up to 58 days and 7 days at 20 °C. Analysis of the expression of ethylene biosynthetic genes (*ACS1*, *ACS2* and *ACO*) revealed a significant induction in chilling-damaged tissue of both varieties that was almost absent in red chilling-tolerant tissue. Similarly, accumulation of transcripts of the ethylene receptors *ETR1* and *ETR3* were also associated with chilling damage, but a cold factor appears to also mediate the expression of these genes. Taken together, our results indicate that high lycopene concentration appears to be responsible for the induction of tolerance to chilling in the red-colored areas of the flavedo of grapefruit during postharvest storage at low temperatures.

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1. Introduction

Grapefruit (*Citrus paradisi* Macf.) are among the most susceptible *Citrus* species to develop CI when stored at temperatures below 8–10 °C (Schirra, 1992; Schirra et al., 1998). CI symptoms in grapefruit are initially manifested as small brown pits on the fruit surface that progressively increase in size, forming shrunken brown spots and depressed areas of different shape, that may affect large surface areas of the rind after prolonged cold storage periods (Schirra, 1992; Schirra et al., 1998; Lafuente and Zacarías, 2006). Susceptibility to development of CI may depend on the grapefruit variety, growing conditions or the harvest season, as it has been demonstrated that grapefruit harvested early and later in the season are more susceptible to CI than mid-season fruit (Schirra et al., 1998; Dou, 2005).

In order to induce cold tolerance in CI-sensitive citrus fruit, various postharvest treatments have been investigated over the years. Temperature conditioning treatments are among the most

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effective systems to induce cold tolerance during low temperature exposure (Hatton and Cubbedge, 1981; Porat et al., 2000; Biolatto et al., 2005). Conditioning treatments in grapefruit are carried out at either 21 °C for 3 days of at 16 °C for 7 days, although the induced tolerance to CI appears to be better with the latter treatment (Hatton and Cubbedge, 1981). Hot water dip treatments (Rodov et al., 1995; Ghasemnezhad et al., 2008), intermitent warming (Davis and Hofmann, 1973), and rinsing and brushing at temperatures of 60 °C for a few seconds (Porat et al., 2000) have been also reported to be effective in inducing chilling tolerance. Moreover, other non-thermal treatments, such as waxing or modified atmosphere packaging (Porat et al., 2000), thiabendazole or imazalil dip application (Schirra et al., 2000) or various combined methods (Rodov et al., 2000) may induce cold stress tolerance, to different extents.

Biochemical and molecular mechanisms involved in chilling-tolerance in *Citrus* fruit have been extensively studied. Genes differentially expressed by temperature-conditioning potentially related to chilling tolerance have been identified in different *Citrus* fruit (Sanchez-Ballesta et al., 2003; Sapitnitskaya et al., 2006; Maul et al., 2008). Results indicated that a complex interplay of different metabolic pathways operate in the induction of cold tolerance (lipid metabolism, metallothioneins, oxidative stress, dehydrins, osmoprotectants, defense responses, among others). Each heat treatment appears to selectively induce the expression of a specific set of genes, and also to modify the expression of chilling-induced transcripts (Maul et al., 2011).

An increased ethylene production during exposure of citrus fruit to chilling temperatures has been observed in many varieties (McCollum and McDonald, 1991; Lafuente et al., 2001; Ghasemnezhad et al., 2008). The increment of ethylene is associated with an induction of *ACO* and *ACS* gene expression during cold storage (Zacarías et al., 2003; Maul et al., 2008) while temperature conditioning treatments down-regulated expression of *ACO* gene (Maul et al., 2011). These results suggest that ethylene is involved in the response of citrus fruit to postharvest cold stress, but whether ethylene is a protective defense response or a cause of CI-induced damage is not fully understood (Lafuente and Zacarías, 2006).

Fruit coloration is one of the most important attributes determining fruit quality. In *Citrus*, fruit color is determined by the specific and particular carotenoid content and composition in the rind and pulp of the different varieties (Kato, 2012; Rodrigo et al., 2013). Carotenoids are C_{40} isoprenoid molecules playing a wide range of functions in plants, transferring energy to chlorophyll, as photoprotectors, dissipating excess light energy, displaying an important role in membrane stabilization (especially in chloro- and chromoplasts), and as powerful scavengers of reactive oxygen species (Britton, 2008). Moreover, carotenoids are also relevant for human nutrition and health, as specific carotenoids are the precursors of vitamin A (α- and β-carotene, and β-cryptoxanthin) and display potent antioxidant properties (lycopene) (Rao and Rao, 2007).

Among carotenoids, lycopene is an acyclic red carotene with 13 double bounds, 11 of which are conjugated and responsible for the high antioxidant capacity of the molecule (Di Mascio et al., 1989; Krinsky, 1989; Aizawa et al., 2011; Rodrigues et al., 2012). Increments in carotenoid content in fruit and vegetables have been associated with a higher tolerance to different stresses. For example, transgenic cells of sweet potato accumulating higher amount of β-carotene were able to grow under stressful salt conditions (Kim et al., 2012, 2013). An increased accumulation of lycopene in tomato has also been associated with a lower incidence of chilling damage (Whitaker, 1994) and damaged fruit showed four times lower lycopene than healthy fruit (Rugkong et al., 2011). Similarly, accumulation of other potent antioxidants (anthocyanins) in transgenic tomato led to a reduction in postharvest deterioration and disease incidence during cold storage (Zhang et al., 2013).

Since the development of CI has been associated with oxidative stress in citrus fruit (Sala, 1998; Sala and Lafuente, 1999) and carotenoids display important antioxidant properties, it is reasonable to conceive that these pigments may have an active role in the protection of fruit from cold stress. Accumulation of lycopene in *Citrus* fruit is an unusual feature, restricted to few species such as grapefruit, pummelo and sweet orange mutants (Liu et al., 2007; Alquezar et al., 2008, 2013; Pan et al., 2009). Thus the objective of the present study was to test the hypothesis that accumulation of lycopene in the rind of grapefruit may protect this tissue from CI development during cold storage. To that end, the incidence of CI was recorded in fruit of two grapefruit varieties with contrasting fruit coloration and carotenoid complement: the white grapefruit Marsh (MSH), that has been demonstrated to be almost devoid of carotenoids in rind and pulp, and the red grapefruit Star Ruby (SR) that is widely recognized by its intense red coloration in the pulp and is also able to develop red color in the rind due to the accumulation of lycopene (Alquezar et al., 2013). Moreover, to corroborate the potential protection to CI induced by this carotene, total carotenoids and especially lycopene content were increased in the rind of grapefruit by chemical (CPTA application) and environmental (fruit shading) manipulation.

2. Materials and methods

2.1. Plant material, treatments and storage conditions

Fruit of the white Marsh and the red Star Ruby grapefruit (*Citrus paradisi* Macf.) were harvested from adult trees from an orchard located in Moncada, Valencia, Spain. Mature fruit were harvested at the end of January, delivered to the laboratory, inspected to be free of damage and defects, selected by uniformity of size and external color and then classified in three replicate lots of 20 fruit each. Fruit from the different experiments were stored at 2 °C and 80–85% RH for up to 58 days with subsequent 1 week storage at 20 °C to simulate shelf-life conditions. At harvest and after 28 and 58 days of storage, the flavedo (colored part of the rind) was excised and immediately frozen in liquid nitrogen, ground to a fine powder and stored at −80 °C until analysis. At each sampling date, rind color was measured using a Minolta CR-330 colorimeter on three locations around the equatorial plane of the fruit, using three replicates of 10 fruit each. Color was expressed as the *a*/*b* Hunter ratio. The *a*/*b* ratio is negative for green fruit, the zero value corresponds to yellow fruit at color break and is positive for orange to red colored fruit. Comparative evaluation of CI incidence in MSH and SR grapefruit harvested from the same orchard was done in two successive seasons.

Two different treatments were performed to induce red coloration in the rind of grapefruit. The first experimental approach was carried out using the inhibitor of lycopene cyclase activity, 2-(4-chlorophenylthio) triethylamine hydrochloride (CPTA), that promotes the accumulation of the red lycopene in plant tissues, including citrus fruit (Coggins et al., 1970; Yokoyama et al., 1972). Fruit of the white MSH grapefruit were harvested in December, delivered to the laboratory and dipped in 5000 mg/L CPTA (Sagechem, China) +0.1 % Triton X-100 solution for 30 s and dried at room temperature. Control fruit were dipped in with the same conditions without CPTA. Control and CPTA-treated fruit were maintained 4 days at 20 °C to stimulate pigment biosynthesis and accumulation, and then stored at 2 °C for up to 58 days and 7 days of shelf-life at 20 °C. In the second approach, SR fruit were covered at the end of July (55 mm diameter) with black plastic bags, to avoid direct sunlight exposure. Uncovered control fruit were located outside of the tree

canopy, adjacent to covered fruit and exposed to normal light conditions. The effect of fruit shading on lycopene content and carotenoid accumulation in red grapefruit has been recently described (Lado et al., unpublished results). Covered (C) and non-covered (NC) fruit were harvested in December, external fruit color was determined and subsequently, stored at 2 °C for 58 days and 7 days of shelf-life at 20 °C. CI incidence, carotenoid composition and ethylene emission were evaluated during storage. The shading experiments were carried out in two orchards, a commercial one located in Lliria (Valencia, Spain) and a second in the *Citrus* Germplasm Bank (Moncada, Valencia, Spain), using at least 60 bagged and non-bagged fruit in each location.

2.2. Chilling injury evaluation

Fruit were inspected for CI incidence (intensity and extension of the damage) and scored on a scale from 0 (no injury) to 3 (severe injury) according to necrotic surface area and browning intensity. Results were expressed as CI index, which was calculated by adding the product of the number of fruit in each category multiplied by the score for each category and afterwards dividing this amount by the total number of fruit evaluated (Schirra et al., 1998). Results correspond to the mean ± S.E. of four replicates of 10 fruit each.

2.3. Carotenoid extraction, identification and quantification

Flavedo pigments were extracted essentially as described previously (Alquezar et al., 2008). Samples were saponified using a 10% methanolic: KOH solution and carotenoid extracts were purified and concentrated to dryness under N_2. Extracts were kept at −20 °C until HPLC analysis. All procedures were carried out on ice under dim light to prevent possible photodegradation, isomerisation and structural changes of carotenoids.

Individual carotenoid composition of each sample was analyzed by HPLC with a Waters liquid chromatography system equipped with a 600 E pump and a model 2998 photodiode array detector, and Empower software (Waters). A C30 carotenoid column (250 × 4.6 mm, 5 μm) coupled to a C30 guard column (20 × 4.0 mm, 5 μm) (YMC Europe GMBH) was used. Samples were prepared for HPLC by dissolving the dried carotenoid extracts in $CHCl_3$: MeOH: acetone (3:2:1, v:v:v). A ternary gradient elution with MeOH, water and methyl tert-butyl ether (MTBE) was used for carotenoid separation as reported in previous work (Alquezar et al., 2008). The carotenoid peaks were integrated at their individual maxima wavelength and their content was calculated using the following calibration curves: β-cryptoxanthin (Extrasynthese); lutein (Sigma) for lutein and violaxanthin isomers; lycopene (Sigma); zeaxanthin (Extrasynthese); β-carotene (Sigma); 8-β-apocarotenal (Roche) for β-citraurin. Standards for calibration curves of phytoene, phytofluene and ζ-carotene were obtained from purified flavedo extracts of 'Pinalate' sweet orange fruit, which accumulate large amounts of these compounds (Rodrigo et al., 2003). Total carotenoid content was calculated as the sum of individual carotenoids. Samples were extracted twice and each analytical determination was replicated three times. All operations were carried out on ice under dim light to prevent photodegradation, isomerisation and structural changes of carotenoids.

2.4. Determination of ethylene production

Ethylene production was measured at different time intervals during storage of MSH and SR grapefruit at 2 °C. Three replicates of four fruit each were incubated in hermetically sealed jars for 5 h and maintained at the same storage temperature. After that period, 1 mL of air from the headspace was withdrawal with a hypodermic syringe and the ethylene content analyzed by GC. A PerkinElmer Autosystem (Norwalk, CT) gas chromatograph equipped with an activated alumina column and an ionization flame detector was used. The detector was stabilized at 180 °C, the injector and column temperature was settled at 140 °C and nitrogen was used as gas carrier. Results were expressed as the mean of three replicates ± S.E.

2.5. Quantitative real time-PCR analysis

Total RNA was isolated from the flavedo of fruit sampled at harvest and after 28 and 58 days at 2 °C, using RNeasy Plant Mini Kit (Qiagen) and subsequently treated with DNase (DNA free, DNase treatment & removal, Ambion). The transcripts present in 2 μg of total RNA were reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen) in a total volume of 20 μL. One μL of a 5-times diluted first-strand cDNA, containing approximately 100 ng of cDNA, was used for each amplification reaction. Quantitative real-time PCR was performed on a LightCycler 480 instrument (Roche), using the LightCycler 480 SYBRGreen I Master kit (Roche). Reaction mix and conditions followed the manufacturer's instructions. The primers employed for the amplification of each gene related to ethylene biosynthesis and perception (*ACS*-ACC synthase, *ACO*-ACC oxidase, *ETR*- ethylene response factor) are described in Alós et al. (2014). The protocol for all genes analyzed consisted of 10 min at 95 °C for pre-incubation, then 40 cycles of 10 s 95 °C for denaturation, 10 s at 59 °C for annealing and 10 s at 72 °C for extension. Fluorescent intensity data were acquired during the extension time. For expression measurements, we used the LightCycler 480 Software release 1.5.0, version 1.5.0.39 (Roche) and calculated expression levels relative to values of a reference sample using the Relative Expression Software Tool (Pfaffl et al., 2002). Normalization was performed using the expression levels of the *ACTIN* gene which primers were described in Alós et al. (2014). For all genes analyzed, the reference sample was the expression value obtained in the flavedo from NC fruit after 58 days of storage at 2 °C which was set at 1. Results are the average of four independent replicates.

2.6. Transmission electron microscopy (TEM)

Samples preparation for microscopy was performed at the Central Service for Support to Experimental Research (SCSIE, Universitat de Valencia). The flavedo samples were manually cut in small squares of 5 mm long × 1 mm wide, being fixed in Karnousky solution (0.5 % glutaraldehyde, 2.5 % paraformaldehyde and phosphate buffer 0.1 M pH 7.4) and washed afterwards two times for 10 min in PIPES buffer 0.1 M (pH 7.2). Then, samples were post-fixed for 1 h in osmium tetroxide (1 % tetroxide at 50 % in PIPES buffer 0.1 M) and washed for 20 min with PIPES and briefly rinsed in water. Samples were stained in uranyl acetate (2 % aqueous solution) for 20 min, proceeding afterwards to dehydrate the tissue, submerging the sample in successive 10 min immersions of ethanol 30–50–70–95 % and absolute ethanol at the end. After dehydration, the samples were embedded in SPURR resin, following a gradual procedure consisting of 10 min in acetonitrile for 10 min, 50:50 acetonitrile:resin overnight and finally pure resin for 6 h. The samples were placed in capsules with SPURR resin and polymerized for 20–24 h at 60 °C. Once polymerized, the ultra-thin (60–150 μm) sections were cut in a Leica microtome (OmU3, Reichert, Austria). Sections were stained for 5 min with lead citrate (Reynold's), afterwards proceeding to visualization in a transmission electron microscope (Hitachi H7000, Japan).

2.7. Experimental design and statistical analysis

The experimental design was completely randomized. Results were expressed as mean ± standard errors (SE) and for continuous

variables with normal distribution, data were subjected to analysis of variance and differences between means determined by Tukey test, setting significance level at $p < 0.05$. In the case of gene expression analysis, data were analyzed using the software REST 2009 (http://genequantification.de/rest-2009.html) to determine statistical significance. A pair-wise fixed reallocation randomization test was applied, which makes no assumptions about the distribution of the observations.

3. Results

3.1. Incidence of chilling injury and rind ultrastructure during cold storage of Marsh and Star Ruby grapefruit

In order to evaluate the sensitivity of MSH and SR grapefruit to CI damage during cold storage, and whether the red and yellow rind tissue areas in the same SR grapefruit (Fig. 1A and B) showed a differential susceptibility to CI, incidence of the disorder was observed during two consecutive seasons. Both varieties developed CI during cold storage at 2 °C and initial symptoms appeared earlier in MSH (14 days) than in SR (20 days; Fig. 1C and D). The first signs of CI were small shrunken pits in the rind of both grapefruit varieties that spread out on the surface and became dark and brown, forming clusters of sunken areas, affecting external appearance and, therefore, fruit commercial quality (Fig. 1A). CI incidence was similar in MSH grapefruit in the two seasons assayed, but in SR fruit, it was lower in the first season than in the second (Fig. 1C and D). After 7 days of shelf-life at 20 °C, CI damage increased in both varieties, but it was consistently higher in MSH (around 2.0 CI Index) than in SR (about 1.5) in both seasons.

MSH grapefruit were of uniform pale-yellow coloration throughout the whole fruit surface, whereas those of SR developed red areas in the flavedo of variable extension surrounded by yellow zones (Fig. 1A). One of the most remarkable features of the comparison in CI development between MSH and SR was that while MSH developed CI symptoms homogeneously distributed throughout the whole fruit surface, in SR fruit lesions were restricted to the yellow zones whereas the red zones remained undamaged during the entire storage period (Fig. 1B). This effect was consistently observed in fruit of the different replicate samples and during the two consecutive seasons.

To confirm the absence of chilling damage in red-colored rind areas of SR grapefruit, an ultrastructural analysis of epidermal cells was performed by TEM (Fig. 2). After 58 days of storage at 2 °C, cells of the red zones of SR were intact, with a well-defined structure and shape, a wide cell wall, undamaged membranes, compact vacuoles filled with content and also intact plastids with electron dense bodies (Fig. 2A). Structure of these epidermal cells remained similar to that of freshly harvested fruit (not shown). By contrast, yellow epidermal cells of the same SR fruit stored at 2 °C, showing visible CI symptoms, were completely different. Cells from these yellow zones were shrunken and collapsed, irregular in shape and the vacuole was contracted and filled with dark material, while other cellular organelles were not clearly defined (Fig. 2B). These cellular cold-induced damages were similar to those observed in the epidermal cells of MSH grapefruit, also of yellow coloration (Fig. 2C). Moreover, damaged tissue showed large empty gaps between cell walls and shrunken membranes. Cell walls from CI damaged epidermal cells were thinner and showed constrictions (asterisks) compared to healthy tissue (Fig. 2B and C).

3.2. Carotenoid content and composition in the flavedo of Marsh and Star Ruby grapefruit

Since tolerance to CI was observed in the flavedo areas of distinctive red color, as compared to the yellow CI-susceptible zones, carotenoid content and composition were determined in MSH, and in the yellow and red flavedo zones of SR. At harvest time, MSH presented an average a/b ratio of −0.09 while this value was of 0.16 and 0.66 in yellow and red rind of SR, respectively (Table 1). Total carotenoid content was also substantially different, accounting for 8.6 µg/g FW in MSH, 55.4 µg/g FW in yellow SR, and more than 120.0 µg/g FW in the red SR flavedo. These differences in carotenoid content were also reflected on carotenoid composition. The rind of MSH contained mainly the xanthophyll violaxanthin (6.0 µg/g FW) and the colorless phytoene (almost 2.0 µg/g FW), and minute amounts of β-carotene and phytofluene (Table 1). In both colored areas of SR grapefruit, phytoene was the most

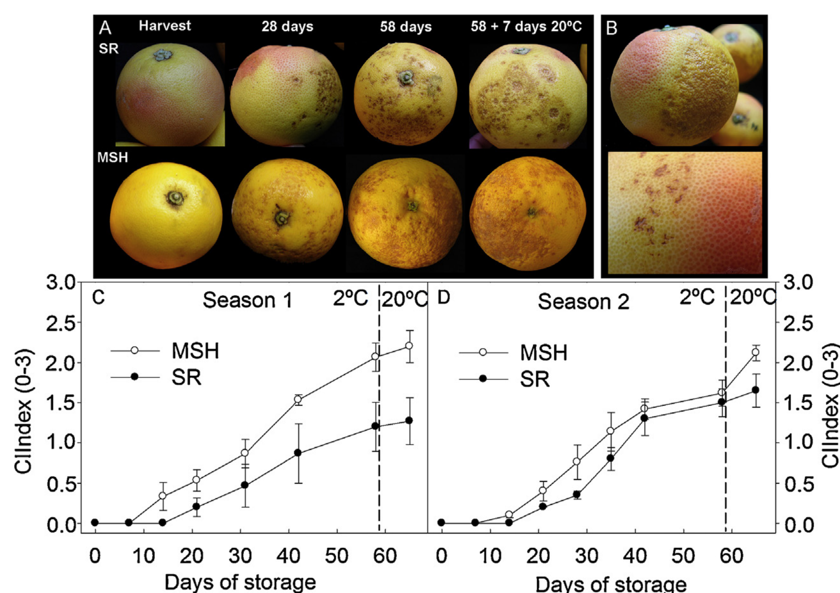

Fig. 1. Morphological development of CI symptoms in the red Star Ruby (SR) and the white Marsh (MSH) grapefruit (A). Magnification of CI symptoms restricted to the yellow areas of the rind in SR grapefruit (B). Evolution of CI index in fruit of MSH and SR grapefruit stored for up to 58 days and 7 days at 20 °C in two consecutive seasons (C and D). Dotted lines show changes on storage temperature.

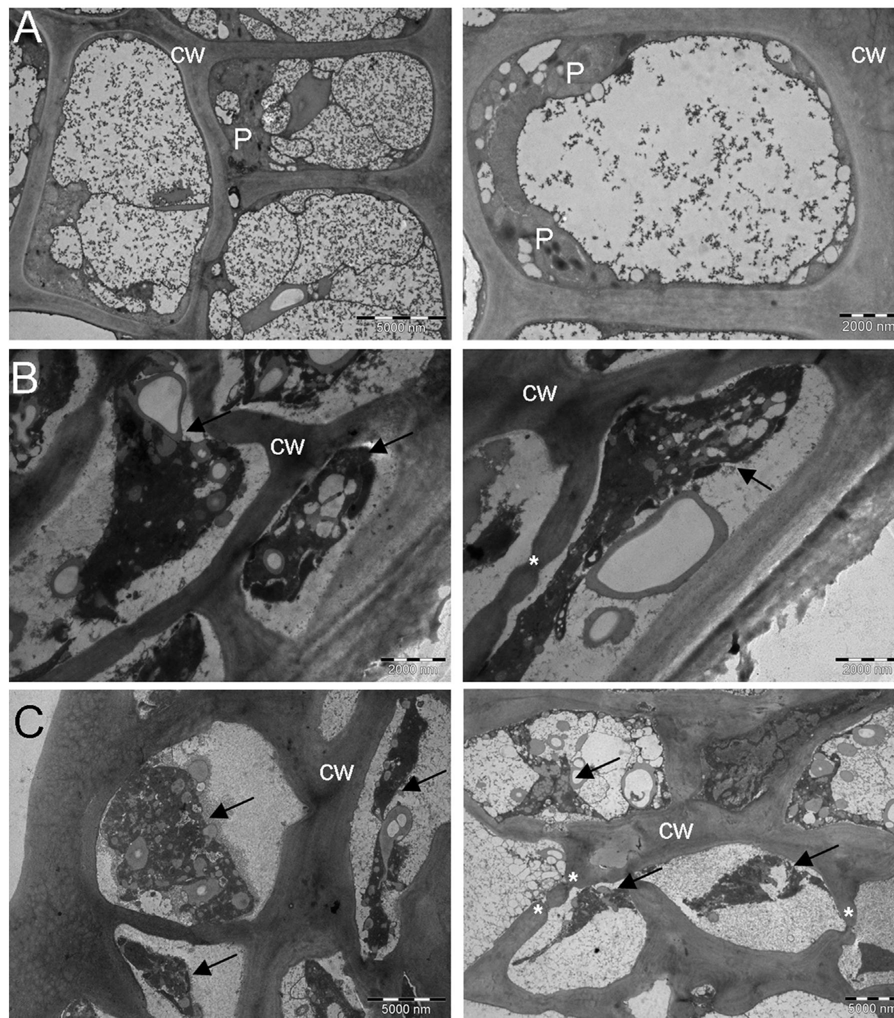

Fig. 2. TEM pictures showing ultrastructure of the epidermal tissue of the red undamaged fruit SR (A), CI-damaged SR (B) and MSH (C) fruit. Initial indicated: cw, cell wall; n, nucleus; P, plastids. Arrows show shrank cells with collapsed membranes where no organelles were detected and asterisks show constrictions in cell wall present in CI damaged cells.

abundant carotenoid, accounting for 74 % of total carotenoids in the yellow zones and nearly for 60 % in the red zones. The most obvious differences were found in lycopene and β-carotene contents between both color areas which were 14- and 4-times higher, respectively, in the red than in yellow flavedo of SR (Table 1). Lycopene and to a lower extent β-carotene, are the carotenoids showing major differences between CI tolerant and sensitive areas in the flavedo of SR grapefruit. Based on these results, two experimental strategies were designed to explore whether these carotenoids, mainly lycopene, may be responsible for this effect.

3.3. Effect of CPTA, a chemical inhibitor of lycopene cyclase activity, on chilling injury and carotenoid content and composition in Marsh grapefruit

CPTA is an inhibitor of lycopene cyclase activity that has been described to provoke lycopene accumulation and, therefore, to induce red coloration in citrus fruit (Yokoyama et al., 1972). Our rationale was that the CPTA-induced accumulation of this carotene would provide tolerance to CI in the chilling-sensitive grapefruit. Application of CPTA to MSH grapefruit caused a rapid red coloration to the rind only 4 days at 20 °C after treatment, color changed from an *a*/*b* ratio lower than 0 to 0.45 (Fig. 3A and B). Control MSH non-treated fruit remained for the same period at 20 °C and no changes in rind color or other alterations were observed. CPTA-treated and non-treated grapefruit were then stored at 2 °C and rind color and CI index were evaluated. Rind color remained nearly constant during storage at 2 °C and slightly increased after simulation of shelf-life at 20 °C (Fig. 3B). CI symptoms appeared later and developed slowly in CPTA-treated than in non-treated control fruit (Fig. 3C). At the end of the storage period and during shelf-life at 20 °C, the CI index was significantly lower in CPTA-treated than in control fruit, reaching values of 1.1 and 1.9, respectively (Fig. 3A and C).

Table 1
Rind color (*a*/*b* ratio) and carotenoids content (μg/g FW) in the rind of the yellow Marsh (MSH) grapefruit and in yellow (yellow SR) and red (red SR) areas of the rind of Star Ruby grapefruit. Data are from fruit at harvest time, and are the same fruit used for experiment of Fig. 1.

	MSH	Yellow SR	Red SR
Color (*a*/*b*)	−0.09 ± 0.02	0.16 ± 0.23	0.66 ± 0.16
Phytoene	1.91 ± 0.30	41.20 ± 3.37	72.3 ± 4.76
Phytofluene	0.11 ± 0.01	5.17 ± 0.09	15.60 ± 1.42
ζ-Carotene	–	1.21 ± 0.17	1.84 ± 0.27
Lycopene	–	1.63 ± 0.47	22.3 ± 2.26
β-carotene	0.14 ± 0.01	1.57 ± 0.09	5.60 ± 0.52
Violaxanthin	6.07 ± 0.15	4.22 ± 0.04	3.32 ± 0.21
Total carotenoids	8.59 ± 0.19	55.40 ± 4.33	120.90 ± 0.55

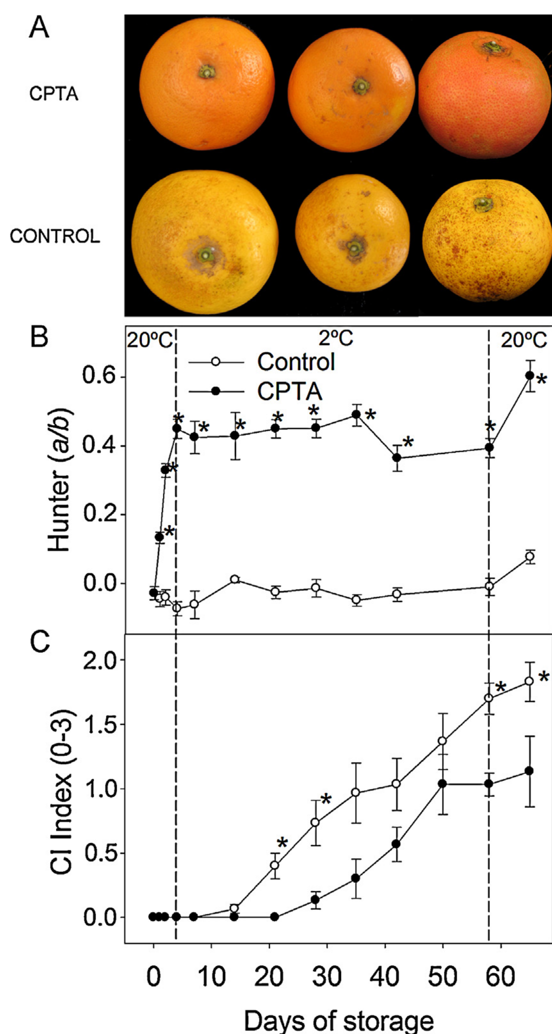

Fig. 3. External appearance of Marsh grapefruit treated or non-treated with CPTA (5000 mg/L) after 28 (right), 58 (middle) and 58 days of storage at 2 °C and 7 days at 20 °C (right) (A). Changes in rind color (*a*/*b* ratio) (B) and CI index (C) in Marsh grapefruit treated and non-treated CPTA (5000 mg/L) and stored at 2 °C for 58 days and 7 days at 20 °C. Dotted lines show changes on storage temperature. *asterisks indicate differences between CPTA treated and non-treated fruit at each sampling date ($p < 0.05$, Tukey).

As expected, CPTA substantially modified carotenoid content and composition. After four days of treatment at 20 °C, total carotenoid content increased more than 7-times in CPTA-treated fruit (from 6.3 to 48.0 μg/g FW), becoming 13-times higher than in control fruit (3.7 μg/g FW), which carotenoid levels even decreased during that period (Table 2). Linear carotenes experienced the more relevant increment induced by CPTA (Table 2), as phytoene increased 9-times and especially lycopene which was 32-times higher than in non-treated fruit (Table 2). The content of carotenoids downstream of lycopene, such as xanthophylls, remained almost constant in CPTA-treated fruit but was reduced in control grapefruit (Table 2).

3.4. Incidence of CI, carotenoid content and composition in shaded and non-shaded fruit of Star Ruby grapefruit

Fruit of the red SR usually develop an intense external red coloration upon shading on the tree (Lado et al., unpublished results). This effect was used to induce red coloration and to increase lycopene content in the rind in order to evaluate if these fruit presented an increased tolerance to cold-induced damage. Experiments were carried out in two locations (Lliria and *Citrus* Germplasm Bank, IVIA, Moncada, Spain), under different climatic and field conditions. Fruit were bagged in July (immature green stage) and harvested in December. At harvest time, the *a*/*b* ratio was 0.14 in non-covered (NC) fruit and near 1.0 in covered (C) ones, corresponding to yellow and red coloration, respectively (Fig. 4, Table 3).

Incidence of CI was similar in fruit from both locations but significantly lower in coloured than in NC fruit. CI symptoms appeared in NC fruit after 20 days of cold storage, whereas in C fruit were delayed until 40 days. Moreover, severity of the damage was higher in NC fruit, reaching a CI index higher than 2 at the end of storage in both locations. Fruit C never exceed a CI index value of 1, even after 7 days of shelf-life at 20 °C, being commercially acceptable during all the storage period (Fig. 4B and C).

Fruit shading caused profound changes in carotenoid content and composition and results from both locations were similar (Table 3). Total carotenoids in the rind were nearly 5-times higher in C fruit than in NC fruit. Shaded fruit presented higher carotene content which accounted for more than 96 % of total carotenoids in the rind, being phytoene (38–49 %) and lycopene (38–53 %) the more relevant. In accordance with the intense red color of C fruit, lycopene experienced the most remarkable increment, since in the rind of NC fruit did not exceed 0.6 μg/g FW compared to 42–43 μg/g FW in C fruit. The concentration of β-carotene was also higher in the rind of C fruit, but in contrast, xanthophylls concentration was reduced (Table 3).

3.5. Ethylene production and expression of ethylene biosynthetic and receptors genes during cold storage of Marsh and Star Ruby grapefruit

Since ethylene has been described to play a role in CI development in grapefruit, emission of ethylene and transcriptional changes in biosynthetic (ACC synthase-*ACS1* and *ACS2* and ACC oxidase-*ACO*) and receptors (*ETR1, ETR2* and *ETR3*) genes were evaluated during storage of MSH and of NC (that developed CI) and C (tolerant to the disorder) SR fruit (Fig. 5). Ethylene production was similar in all fruit examined up to 25 days of cold storage (Fig. 5A). After that period, ethylene production was higher in fruit developing CI than in tolerant C fruit. During 7 days of shelf-life at 20 °C, ethylene production increased and was more than twice in CI-damaged fruit (MSH and NC SR) than in those tolerant to CI (C SR fruit, Fig. 5A).

The changes in the expression of ethylene biosynthetic genes during storage at 2 °C were in agreement with the changes in ethylene production. *ACS1*, *ACS2* and *ACO* transcripts markedly

Table 2
Effect of CPTA application (5000 mg/L) on carotenoid content in the rind of MSH grapefruit. Fruit were treated with the CPTA, an inhibitor of lycopene cyclase activity, and incubated at 20 °C for 4 days and after that period, rind tissue was collected for analysis of carotenoid content and composition. Carotenoid concentration (μg/g FW) is the mean ± S.E. of three determinations. Fruit were the same as used in experiment described in Fig. 3.

		Treatment	
Carotenoids	Harvest	Control	CPTA
Phytoene	1.14 ± 0.19	0.97 ± 0.03	9.18 ± 1.41
Phytofluene	–	tr	1.03 ± 0.28
ζ-Carotene	–	–	0.17 ± 0.06
Lycopene	–	–	32.20 ± 1.15
β-citraurin	0.21 ± 0.03	tr	0.16 ± 0.02
Zeaxanthin	0.07 ± 0.01	–	–
Violaxanthin	4.25 ± 0.12	2.38 ± 0.31	4.18 ± 0.13
Lutein	0.16 ± 0.01	0.11 ± 0.04	0.18 ± 0.02
Total carotenoids	6.35 ± 0.13	3.73 ± 0.37	48.00 ± 2.96

tr < 0.05 μg/g FW.

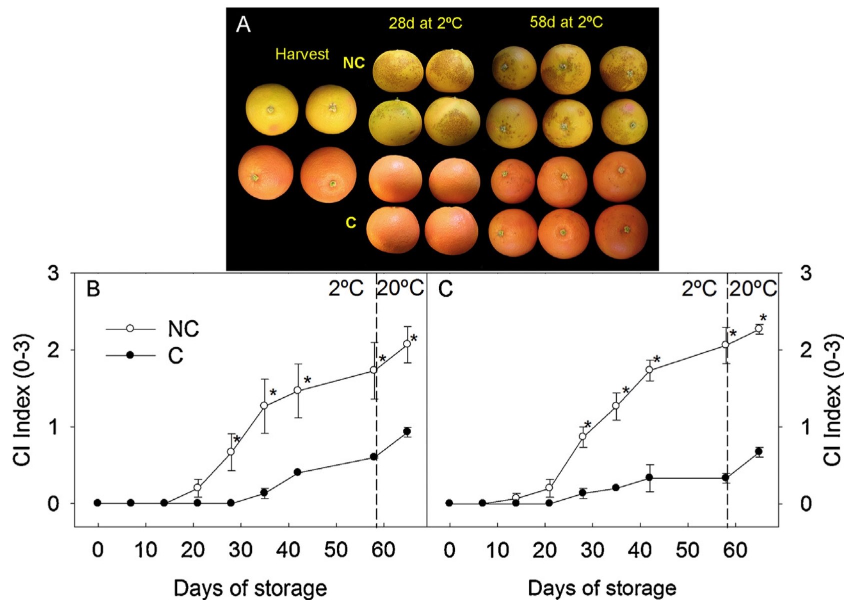

Fig. 4. External appearance of Star Ruby fruit covered (C) or non-covered (NC) on the field at the harvest time and after 28 and 58 days of storage at 2 °C (A). Changes in CI index in C and NC SR grapefruit harvested form location 1 (B, Lliria) or location 2 (C, IVIA) and stored at 2 °C for 58 days and 7 days at 20 °C. Dotted lines show changes on storage temperature. *asterisks indicate differences between NC and C fruit at each sampling date ($p < 0.05$, Tukey).

increased after storage at 2 °C in the rind of fruit developing CI. The major increments were registered in the accumulation of *ACO* transcripts followed by *ACS1* and *ACS2* in MSH grapefruit which was several times higher than in the NC CI-sensitive SR (Fig. 5B). It is noticeable that in the red rind of CI-tolerant SR (C fruit), abundance of the *ACS1*, *ACS2* and *ACO* transcripts was negligible as compared with the rind of sensitive fruit, indicating that stimulation of ethylene biosynthetic genes is a chilling response (Fig. 5B).

The relative variation in the expression of *ETR* genes was lower than that of ethylene biosynthetic genes. In general, *ETR* genes were up-regulated during cold storage in the three types of fruit evaluated. Stimulation of *ETR1* and *ETR3* genes was in accordance with the severity of cold-induced damage, with a lower expression in the rind of C fruit. However, *ETR2* was more expressed in NC SR than in the white MSH (Fig. 5C). It is interesting to note that a slight but significant increment in the expression of *ETR1* and *ETR3* in response to cold was detected in the rind of the CI-tolerant SR (C fruit), which may indicate a contribution of cold in the induction of these *ETR* genes (Fig. 5C).

4. Discussion

Citrus fruit quality is highly determined by external appearance, which directly influences marketability and consumer acceptance. Maintenance of high quality during transport, storage and extended postharvest life of citrus fruit is currently based on the use of low temperatures. However, some citrus species are highly sensitive to cold and develop CI symptoms in the rind. The white Marsh and the red Star Ruby grapefruit varieties are among the most cold-susceptible citrus varieties and develop CI both in the field and during postharvest cold storage (Schirra et al., 1998; Dou, 2005; Sapitnitskaya et al., 2006). CI symptoms in our study were apparent after 14 days storage at 2 °C in MSH grapefruit and slightly later in SR (Fig. 1). These differences in the initiation and development of CI symptoms may be due to seasonal variations and also to varietal susceptibility to CI, but in general, chilling symptoms were similar to those previously observed in fruit of the same varieties growing in different countries and under distinct environmental conditions and cultural practices (McCollum and MacDonald, 1991; Schirra, 1992, 1998; Maul et al., 2008, 2011). However, comparison of chilling response in fruit of MSH and SR grapefruit revealed a striking difference between the two varieties; while chilling symptoms were distributed uniformly throughout the whole rind surface of MSH fruit, in SR grapefruit were restricted to the yellow areas and the red sections were almost completely absent of damage (Fig. 1). This particular and irregular distribution of CI symptoms was consistently observed in fruit of SR harvested from different orchards and also independently of the extension of the red blotches. It is remarkable that we also observed absence of CI in red zones of SR fruit harvested earlier on the season and when the surrounding rind was still greenish (data not shown). From these observations we concluded that the red zones of SR grapefruit showed a higher tolerance to CI during postharvest storage at low temperature.

The tolerance to CI in the red areas of SR was confirmed by ultrastructural TEM observations. Epidermal cells of red chilling-tolerant tissue presented a normal morphology with a defined membrane, an intact typical vacuole and well developed plastids

Table 3
External color (a/b ratio) and carotenoid content in the rind (μg/g FW) in covered (C) and non-covered (NC) Star Ruby grapefruit. Fruit from two locations were covered by black plastic bags in July or remained uncovered, and harvested in December. Data are from fruit at the harvest time and the mean of three determinations ± S.E.

	Location and treatment			
	Lliria		IVIA	
	NC	C	NC	C
Color (a/b)	0.14 ± 0.02	0.94 ± 0.04	0.13 ± 0.02	1.00 ± 0.07
Phytoene	13.40 ± 0.27	31.80 ± 1.82	21.90 ± 0.04	55.80 ± 2.35
Phytofluene	0.97 ± 0.02	6.59 ± 0.45	2.18 ± 0.14	14.30 ± 0.92
ζ-Carotene	–	0.17 ± 0.04	tr	0.46 ± 0.28
Lycopene	0.58 ± 0.08	43.90 ± 1.93	0.04 ± 0.00	42.40 ± 1.36
β-carotene	0.48 ± 0.02	2.58 ± 0.11	0.44 ± 0.22	3.51 ± 0.58
Zeaxanthin	0.11 ± 0.01	–	0.11 ± 0.01	–
Violaxanthin	3.14 ± 0.16	0.48 ± 0.08	1.26 ± 0.29	0.57 ± 0.20
Lutein	0.52 ± 0.04	0.01 ± 0.01	0.45 ± 0.02	–
Total carotenoids	19.20 ± 0.22	85.60 ± 4.12	26.80 ± 0.77	117.00 ± 2.10

tr < 0.05 μg/g FW.

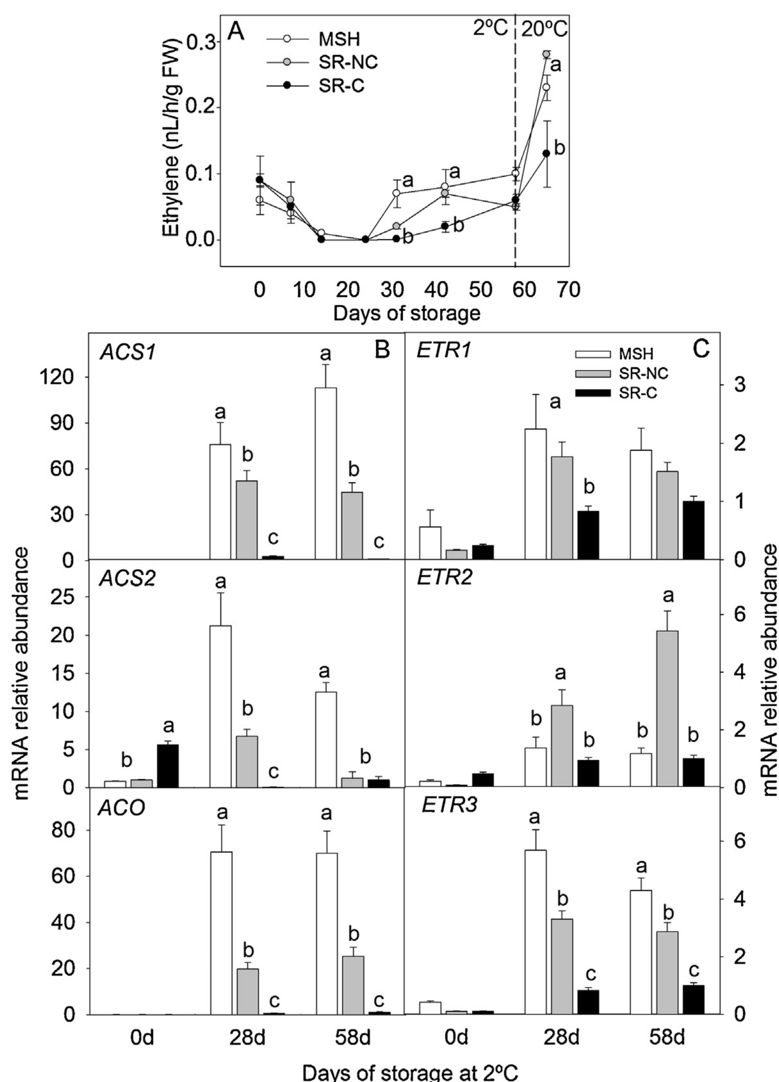

Fig. 5. Ethylene production (nL/g FW/h) in Marsh (MSH) and non-covered (NC) and covered (C) Star Ruby (SR) grapefruit stored for up to 58 days and 7 days at 20 °C (A). Accumulation of ethylene biosynthetic genes (*ACS1*,*ACS2*, *ACO*) (B) and ethylene receptors (*ETR1*, *ETR2* and *ETR3*) (C) in the flavedo of MSH and NC and C SR grapefruit stored 28 and 58 days at 2 °C. Dotted lines show changes on storage temperature. *different letters indicate significant differences between means at each sampling date ($p < 0.05$, Tukey test for ethylene and pair wise randomization test for gene expression). The absence of letters indicates no significant differences.

(Fig. 2A). These structures are characteristic of healthy tissue and denoted absence of chilling damage after prolonged storage at 2 °C. By contrast, yellow areas of fruit from both varieties stored for 58 days under the same temperature showed evident CI symptoms, characterized by membrane shrinkage and loss of cell integrity, and undefined organelles (Fig. 2B and C). Cell wall damage was also evidenced by constriction sites that became narrower compared to intact walls of healthy cells (Fig. 2). Similar ultrastructural changes have been previously described the rind of MSH, where low temperature induced damage of epidermal cells and several layers of the mesophyll cells below. An increment in lipid material inside the cytoplasm and the vacuole together with cytoplasm degradation and collapse were also described as deleterious damage in grapefruit under cold stress (Platt-Aloia and Thomson, 1976). In our TEM observations, a clear separation of the internal membrane from the cell wall was shown in cells of rind-damaged grapefruit (Fig. 2B and C), a defect that has been also described in chilling-damaged eggplant and was triggered by the contraction of the cytoplasm and plasmatic membrane (Concellón et al., 2007). These morphological alterations in cell walls of cold-damaged tissue may be due to the changes in the composition of cell wall that would provoke weakness and increase its permeability, favoring then membrane damage and finally leading to cell death. The maintenance of cell wall structure and its components (mainly higher water soluble pectins and calcium bridges) are associated with the tolerance to CI induced by high-temperature conditioning in the chilling-sensitive 'Fortune' mandarin (Holland et al., 2012), reinforcing the importance of cell wall composition on membrane integrity in the cold-induced damage. Absence of visible damage and weakness in cell wall of cells from red tissue of cold-stored SR grapefruit provides additional evidence of the resistance of this tissue to cold damage.

Changes in carotenoid content and composition are probably the most obvious differences between yellow chilling-sensitive and red chilling-tolerant rind tissue. It should be noted that the yellow tissue of the white MSH grapefruit was almost devoid of carotenoids (Table 1), as previously described (Alquezar et al., 2013), but the corresponding yellow tissue of SR have a high carotenoid content in which nearby 75 % of the total was the colorless phytoene, and lycopene accounted for only 3 % (Table 1). These differences in carotenoid content may probably be associated with the different susceptibility of both grapefruit varieties to CI, as MSH is recognized by a higher sensitivity to develop CI than red grapefruit (Dou, 2005) that was also evidenced

in our study (Fig. 1). Despite the relatively high carotenoid content detected in the yellow rind of SR grapefruit, the tissue developed chilling damage that was almost indistinguishable to that occurring in the yellow rind of MSH. These results indicate that total carotenoid content appears not to be the main factor responsible for the induction of tolerance to CI in red zones of SR, being more likely related to the carotenoid composition and, specifically, to lycopene concentration. The content of this linear carotene was 14-times higher in the red than in yellow zones of SR grapefruit and it was completely absent in the rind of MSH (Table 1). Thus, our working hypothesis was addressed to establish a potential relationship between lycopene content and tolerance to CI in red tissue of SR grapefruit. We reasoned that if by chemical manipulation of carotenoid biosynthesis, through application of CPTA, an inhibitor of β-lycopene cyclase enzyme (Coggins et al., 1970; Yokoyama et al., 1972), the lycopene content could be increased in MSH grapefruit, it would enhance the tolerance to CI. As a result, CPTA-treated MSH grapefruit developed an intense red coloration, and the rind accumulated 13-times more total carotenoids than non-treated fruit and a relative high content of lycopene (32 μg/g FW) which was absent in control ones (Table 2). As expected, this increment in lycopene content was associated with a significant delay in the initiation and a reduction of chilling damage in the chilling-sensitive MSH grapefruit (Fig. 3). It is interesting that despite being different varieties, total carotenoids in CPTA-treated MSH fruit were lower than in yellow areas of SR (Table 1), but lycopene content was almost 20-times higher, suggesting the accumulation of lycopene above a threshold may be a key factor in the induction of tolerance to chilling.

In a second approach, we were able to significantly enhance chilling tolerance in SR grapefruit by fruit bagging. Light avoidance in SR grapefruit modifies expression of carotenoid biosynthetic genes and stimulated lycopene accumulation (Lado et al., unpublished results). By this environmental manipulation during fruit development, lycopene was increased as much as 40 μg/g FW, accounting between 36–52 % of total carotenoids in the rind of SR fruit, which is a massive amount of lycopene and a unique feature among *Citrus* fruit (Rodrigo et al., 2013). The concentration of linear carotenes, phytoene and phytofluene as well as of the cyclic carotenoid β-carotene, was also between 2–8 times higher in bagged than in non-bagged SR fruit (Table 3). Although these experimental strategies may provoke other unknown effects on fruit physiology, the changes on carotenoid concentration confirmed our rationale and reinforced the concept that the increased tolerance to CI in red rind of grapefruit could be related to high carotene content, mainly lycopene.

Elevated lycopene content has been linked to lower CI incidence in tomato during cold storage (Whitaker, 1994) and chilling stress was described to reduce lycopene content in tomato (Rugkong et al., 2011), establishing then a possible linkage between these two factors. In citrus fruit, cold stress has been associated with an oxidative burst (Sala, 1998; Sala and Lafuente, 2000) and carotenoids, specially lycopene, display a powerful antioxidant activity (Krinsky, 1989; Aizawa et al., 2011) and ROS scavenging ability, then modulating redox signaling (Palozza et al., 2012). It is reasonable to assume that the tolerance to CI injury observed in the red areas of grapefruit could be due to an increased antioxidant capacity induced by the high lycopene concentration in that tissue. The experimental treatments designed to increase lycopene content also promoted accumulation of upstream linear carotenes, such as phytoene or phytofluene (Tables 2 and 3). Then, we cannot discard that altogether these increments in linear carotenes could participate in the induction of tolerance to CI in grapefruit. In this context, 'Pinalate', a sweet orange mutant with elevated amounts of phytoene, phytofluene and ζ-carotene, was more tolerant to CI than the ordinary sweet orange fruit (Alférez et al., 2005).

A partial contribution of β-carotene cannot be ruled out since this cyclic carotene also displays antioxidant activity (Aizawa et al., 2011) and its content was slightly enhanced in bagged fruit (Table 3). Moreover, the localization of β-carotene inside membranes could favor a higher fluidity during low temperature stress, promoting cold acclimation and therefore, favoring CI tolerance. Besides their well-known function as light collectors and photoprotectors, carotenoids are instrumental as structural components for the stabilization of the three-dimensional integrity of membranes under potentially harmful environmental conditions (Havaux, 1998).

Evidence from transgenic strategies reinforces the role of carotenoids as oxidative stress scavengers. Induction of high carotenoid accumulation in sweet potato calli (Kim et al., 2013) or cultured cells (Kim et al., 2012) conferred resistance to salt-induced oxidative stress. The over-expression of different enzymes of carotenoid biosynthesis (phytoene synthase-PSY and lycopene cyclase-βLCY) in *Arabidopsis,* determined a higher accumulation of carotenoids and also an increased resistance to salt-mediated oxidative stress (Han et al., 2008; Chen et al., 2011). Furthermore, other powerful antioxidant molecules, such as anthocyanins, may protect fruit from postharvest stress conditions and pathogen infection (Zhang et al., 2013). Similarly, increasing ascorbic acid in tomato fruit was also associated with a higher chilling tolerance (El Airaj et al., 2013). Taken together, carotenoids and CI data in MSH and SR grapefruit suggest that the presence of higher levels of these antioxidants, mainly lycopene, could favor rind tolerance to oxidative stress induced by cold.

Analysis of the expression of ethylene biosynthetic and receptor genes in the rind of grapefruit varieties with contrasting susceptibility to CI has been useful to evaluate the metabolism of the hormone in the response of grapefruit to cold temperature. The implication of ethylene in fruit chilling stress is still controversial (Lafuente and Zacarías, 2006; Sevillano et al., 2009; Wang et al., 2013). Our results indicate that the ethylene biosynthesis genes analyzed *ACS1*, *ACS2* and *ACO* were clearly induced in relation to the chilling damage (Fig. 5B). These patterns of changes are in agreement with the evolution of ethylene production after 28 days of cold storage that was higher in the rind of fruit developing CI (MSH and non-covered SR) than in red-covered SR (Fig. 5A). *ACS1* isoform showed higher activation than *ACS2* in damaged tissues, suggesting an important role of this isoform in the response of grapefruit to chilling damage. Maul et al. (2008) also found induction of *ACS* and *ACO* by chilling and that each gene was differentially affected by heat conditioning. Our results showed that the three genes of the ethylene biosynthesis analyzed were induced in relation to rind damage which indicates that these genes may be good markers of CI and that their induction during postharvest storage of grapefruit is more likely related to CI than to cold response.

Regarding ethylene perception, expression of three members of the *ETR* gene family revealed a differential and complex regulation of each member, not only by chilling but also by cold. Similar to ethylene biosynthetic genes, *ETR1* and *ETR3* were up-regulated in a chilling-dependent manner (Fig 5C). However, in the red rind of CI-tolerant SR, accumulation of both transcripts was also increased, although to a lower level than in yellow CI-sensitive tissue. This indicates that the expression of *ETR1* and *ETR3* is stimulated in grapefruit by both factors, chilling injury and cold stress. To our knowledge, these are the first results showing changes in ethylene receptor genes during storage of citrus fruit at low temperature and linking ethylene perception with cold stress and chilling damage. Moreover, ethylene receptor genes are up-regulated by exogenous ethylene in other citrus (Katz et al., 2004; John-Karuppiah and Burns, 2010; Alós et al., 2014). It is then plausible that the induction of ethylene biosynthetic genes and ethylene

production during storage of chilling-sensitive fruit at low temperatures may stimulate *ETR* gene expression. Whether ETRs may function during cold storage of citrus fruit as negative regulators of ethylene signaling or stimulating other ethylene-related responses remains to be elucidated (Binder, 2008; Wang et al., 2013).

In summary, our results reveal a positive relationship between carotenes content, mainly lycopene, and chilling tolerance during storage of grapefruit at low temperature. The potential protective role of lycopene in CI has been explored by chemical and environmental stimulation of the content of this carotene in the rind of grapefruit. The antioxidant properties of lycopene are suggested to mediate the tolerance of the red zones to CI. Moreover, expression of ethylene biosynthetic genes appears to be induced in a chilling-dependent manner. However, chilling and also cold participate in the induction of the ethylene receptors genes *ETR1* and *ETR3*.

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Analysis of ethylene biosynthesis and perception during postharvest cold storage of Marsh and Star Ruby grapefruits

Joanna Lado[1,2], María Jesús Rodrigo[1] and Lorenzo Zacarías[1]

Abstract

Grapefruits are among the citrus species more sensitive to cold and develop chilling injury symptoms during prolonged postharvest storage at temperatures lower than 8 °C–10 °C. The plant hormone ethylene has been described either to protect or potentiate chilling injury development in citrus whereas little is known about transcriptional regulation of ethylene biosynthesis, perception and response during cold storage and how the hormone is regulating its own perception and signaling cascade. Then, the objective of the present study was to explore the transcriptional changes in the expression of ethylene biosynthesis, receptors and response genes during cold storage of the white Marsh and the red Star Ruby grapefruits. The effect of the ethylene action inhibitor, 1-MCP, was evaluated to investigate the involvement of ethylene in the regulation of the genes of its own biosynthesis and perception pathway. Ethylene production was very low at the harvest time in fruits of both varieties and experienced only minor changes during storage. By contrast, inhibition of ethylene perception by 1-MCP markedly induced ethylene production, and this increase was highly stimulated during shelf-life at 20 °C, as well as transcription of *ACS* and *ACO*. These results support the auto-inhibitory regulation of ethylene in grapefruits, which acts mainly at the transcriptional level of *ACS* and *ACO* genes. Moreover, *ethylene receptor1* and *ethylene receptor3* were induced by cold while no clear role of ethylene was observed in the induction of ethylene receptors. However, ethylene appears to be implicated in the transcriptional regulation of *ERFs* both under cold storage and shelf-life.

Keywords

Citrus, chilling injury, cold, physiological disorder, ethylene signaling

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INTRODUCTION

Storage at low temperatures is one of the most practical and widely used technologies to maintain quality of fruit and vegetables during postharvest transportation and storage. However, fruits of some citrus species are susceptible to develop chilling injury (CI) symptoms when temperatures drop below 8 °C–10 °C. Fruits of the different grapefruit varieties (*Citrus paradisi* Macf.) and lemon (*Citrus lemons*) are among the most sensitive to develop CI (Lafuente and Zacarias, 2006; Schirra, 1992). CI symptoms in grapefruits are initially manifested as small brown pits on the fruit surface that progressively increase in size forming brown clusters of depressed areas of different shape and that may affect large surface of the flavedo in prolonged cold storage periods (El-Otmani et al., 2011).The sensitivity to CI is variable and depends on the variety and other endogenous and environmental factors. Among relevant grapefruit commercial varieties, the white Marsh Seedless

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(MSH) has been described to be extremely sensitive to cold-induced damage, followed by the red-fleshed Star Ruby (SR) and Red Blush varieties (Schirra et al., 1998). In general, grapefruit harvested early and later on the season are more sensitive to CI than mid-season fruits (Dou, 2005a; Lafuente and Zacarías, 2006).

Despite the susceptibility to CI of grapefruits and other citrus cultivars, storage at temperatures between 1 °C and 2 °C for different periods are required for quarantine treatment to export citrus fruits to several countries, as Japan or USA. Therefore, different methods have been evaluated and proposed to alleviate the severity of chilling damage in sensitive citrus varieties. One of the most studied is the application of high temperatures (37 °C–50 °C) by curing or hot water dips before cold storage, which showed a significant reduction in CI incidence in different sensitive varieties like Fortune mandarin (Lafuente et al., 2011; Sala and Lafuente, 1999), lemon, grapefruits (Rodov et al., 1995), and satsuma mandarins (Ghasemnezhad et al., 2008). As an alternative, application of mild temperatures around 16 °C for one week before storage showed a reduction in CI incidence in different grapefruits (Maul et al., 2008; Maul et al., 2011; Sapitnitskaya et al., 2006). Similarly, intermittent warming (three weeks at 3 °C followed by two weeks at 15 °C) has been described to be highly effective on inducing chilling tolerance in cold-stored fruits (Schirra and Cohen, 1999). However, nowadays the application of high temperatures is not an attractive method to be applied in commercial conditions mainly due to the technological limitations and the high risk to provoke peel damage by the extreme high temperatures (Ghasemnezhad et al., 2008).

The physiological and molecular bases related to the sensitivity of citrus fruits to CI and also to the heat-induced tolerance to CI have been extensively studied over the years. These studies are critical to understand how these processes and treatments are genetically controlled and to provide the basis for further biotechnological manipulation and development of newly successful strategies (Lafuente et al., 2004; Maul et al., 2008). The phytohormone ethylene has been implicated in the plants responses to different biotic and abiotic conditions (Wang et al., 2013). However, the involvement of ethylene in the response of horticultural commodities to cold stress is controversial, and the results indicating either a protective role or causing deleterious effects on CI have been reported (Sevillano et al., 2009). In avocado and plums, application of exogenous ethylene accelerated CI symptoms (Candan et al., 2008), while in nectarines alleviated its development (Zhou et al., 2001). In citrus fruit, ethylene has been related to many stress responses and also was described to be induced under chilling conditions in Marsh grapefruits stored at 5 °C, even before the appearance of CI symptoms (McCollum and McDonald, 1991). In fruits of the hybrid mandarin Fortune, which is very sensitive to develop CI (Lafuente and Zacarías, 2006), exogenous application of ethylene before cold storage led to an increase in the susceptibility to this disorder (Gosalbes et al., 2004; Lafuente et al., 2001). However, a continuous application of the gas at low concentrations during storage in the same variety induced tolerance to chilling (Lafuente et al., 2004), suggesting that ethylene is part of the protective defense mechanisms to maintain fruit natural resistance against various environmental stresses (Marcos et al., 2005; Porat et al., 1999). The response to ethylene appears to be variable and dependent of the variety or the hormone dose applied, as continuous application of different ethylene concentrations during storage of Tahitian lime, Emperor mandarin, Marsh grapefruit, and Valencia oranges at 0 °C resulted in a higher incidence of CI that was proportional to the ethylene dose (Yuen et al., 1995). Moreover, the incidence of CI in Marsh grapefruit was also positively related to the duration of the ethylene treatment before storage (Hatton and Cubbedge, 1981). Similarly, data from the application of the inhibitor of ethylene perception, 1-MCP, reinforce the notion that the role of ethylene in CI may depend on the citrus variety considered. Thus, 1-MCP treatment to Nova and Ortanique varieties significantly reduced CI symptoms after 60 days at 1 °C (Salvador et al., 2006) as also occurred in Murcott (Edagi et al., 2010) and Fallglo (Dou et al., 2005b). By contrast, fruits of the chilling-sensitive Fortune mandarin treated with 1-MCP developed faster and higher CI during storage at 2 °C, suggesting that in this variety as well as in oranges, ethylene is also part of the defense mechanism developed to cope with cold stress (Lafuente et al., 2001; Marcos et al., 2005; Porat et al., 1999).

The transcriptional regulation of ethylene biosynthesis and perception has been also described to be affected by chilling conditions in different fruits. Early studies in Passe-Crassane pears, a cultivar that require exposition to low temperature to ripen, demonstrated an accumulation of *ACS* and *ACO* transcripts as well as of ACO protein during postharvest chilling treatment. After transference to shelf-life conditions, ACO protein was able to oxidize ACC and then to increase ethylene production, stimulating pear fruit ripening (Lelièvre et al., 1997). Ethylene biosynthetic genes (ACC synthase-*ACS* and ACC oxidase-*ACO*) as well as ethylene receptors (*ETRs*) transcripts were induced during cold stress in the field in climacteric avocado fruit (Hershkovitz et al., 2009). In tomato fruit, *ACS* gene expression showed a transient increase after one week of cold storage, while *ETR* expression pattern was very variable and strictly depended on the gene isoform evaluated (Rugkong

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et al., 2011). A marked induction in *ETR1* expression was registered in loquat fruit stored at low stressful temperatures, being directly correlated with CI development; however, other isoforms showed different patterns of expression in response to cold stress (Wang et al., 2010).

In citrus fruit, previous studies have evidenced an increase in ethylene production during postharvest storage at low temperatures (Cooper et al., 1969; McCollum and McDonald, 1991; Schirra, 1992, Lafuente et al., 2001). Regulation of this cold-induced ethylene production is still not well understood, and it appears that the response of the different varieties may be different and also dependent on the susceptibility to CI. In the peel of orange fruits, Wong et al. (1999) observed the transcript accumulation of one *ACS* isoform that appears to be dependent on the time of storage under cold conditions. Large-scale analysis of gene expression in cold stored and heat-conditioning grapefruits has revealed accumulation of *ACO* gene transcript (Maul et al., 2008; Zhu et al., 2011). In the chilling-sensitive Fortune mandarin, it has been observed an increase in ethylene production during storage at 2 °C concurrently to the development of CI symptoms (Lafuente et al., 2001). Moreover, in the peel of this mandarin *ACS2* and *ACO* transcripts, as well as ACO protein and the ethylene metabolic precursor ACC, were accumulated during the chilling period. Therefore, upon transference to shelf-life at 20 °C, there was an overstimulation of ethylene production at the expenses of the pre-formed ACO protein and the substrate ACC (Zacarías et al., 2003). This regulation of ethylene biosynthesis under cold stress may explain the rapid and massive increase in ethylene production usually detected in fruits upon transference to shelf-life, at temperatures near the optimum for enzyme activity. However, our understanding about how ethylene biosynthesis is regulated by cold stress and how these changes are coordinated with ethylene perception and responses to these postharvest conditions in citrus fruits are still unknown. Then, the objective of the present study was to explore the transcriptional changes in the expression of ethylene biosynthesis, receptors and response genes during cold storage of the white Marsh and the red Star Ruby grapefruits. The effect of the ethylene action inhibitor, 1-MCP, was also evaluated to ascertain the involvement of ethylene in the regulation of the genes of its biosynthesis and perception pathway.

MATERIALS AND METHODS

Plant material, treatments and storage conditions

Fruits of grapefruit (*Citrus paradisi*) cv. Marsh and cv. Star Ruby from adult trees cultivated under commercial conditions in an orchard located in Moncada, Valencia (Spain), were harvested in January at full maturity. Fruits were delivered to the laboratory, inspected for free of injuries or defects, selected by uniformity, and divided into replicated samples. Replicate lots of fruits were treated with 1 μL/L of the inhibitor of ethylene action 1-MCP, as described in Lafuente et al. (2001), or remained in a continuous air flow for 16 h at 20 °C and 90 % RH. After the treatment, fruits were ventilated and stored at 2 °C (chilling temperature) or 12 °C (non-chilling control temperature), and 80–85 % RH for up to 58 days. 1-MCP treated fruits were stored only at 2 °C. After 58 days of storage at 2 °C, replicate samples of fruits were transferred for seven days at 20 °C to simulate a shelf-life period. During postharvest storage and shelf-life, CI incidence and ethylene production were determined in cold stored and control fruit. At each sampling date (0, 24 and 58 days, and 58 days + 7 days at 20 °C), flavedo tissue was excised from the whole fruit, frozen in liquid nitrogen, ground to a fine powder and stored at −80 °C until analysis.

Estimation of CI index

Fruits were inspected for the intensity and extension of chilling damage and rated on a scale from 0 (no damage) to 3 (severe damage) (Schirra et al., 1998). The results were expressed as peel damage index that was calculated according to the formula

$$\Sigma(\text{Peel damage scale } (0-3) \times \text{number of fruit within each class}) / \text{total number of fruit.}$$

Three replicates of 10 fruits per treatment, temperature, and grapefruit variety were used for CI evaluation using a complete randomized design.

Determination of ethylene production

Ethylene production was determined by incubating three replicates of three fruits each into 4 L flasks that were hermetically sealed and maintained at the storage temperature. After 3 h of incubation, 1 mL of air samples from the headspace of the flasks was withdrawn with a hypodermic syringe and injected into a gas chromatograph (Perkin Elmer Autosample) equipped with a flame ionization detector and an activated alumina column. Nitrogen was used as carrier gas, and the temperature of the column was maintained at 140 °C. Ethylene production values represent the mean of three replicates and for statistical significance an analysis of variance test was performed and Tukey test applied for mean separation ($p \leq 0.05$).

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Quantitative real time-PCR

Total RNA was isolated from the flavedo of fruit at each sampling date, using RNeasy Plant Mini Kit (Qiagen) and subsequently treated with DNase (DNA free, DNase treatment and removal, Ambion). The procedure followed for relative gene expression quantification and primers used for gene amplification as well as software details are described in Alós et al. (2014a). Normalization was performed using the expression levels of the *Actin* gene as reported in Alós et al. (2014b). For each gene, a relative value of 1 was assigned to the expression determined in the flavedo of SR grapefruit at harvest time. The results were the average of four independent replicates.

RESULTS AND DISCUSSION

To understand the regulation of ethylene biosynthesis and perception during cold storage of grapefruits, matured fruits of the white Marsh and the red Star Ruby, both very sensitive to develop CI (El-Otmani et al., 2011), were stored at 2 °C for up two months, followed by a simulation of shelf-life period for seven days at 20 °C. Evolution of ethylene production and CI index was followed in fruits of both varieties (Figure 1). To understand the involvement of ethylene in its own synthesis and the potential effect on CI development, grapefruits were pre-treated with the inhibitor of ethylene action, 1-MCP (Watkins, 2006) and then stored at 2 °C.

In MSH and SR grapefruits, CI symptoms appeared after 14 days of storage at 2 °C in both 1-MCP treated and control fruit, while fruits stored at 12 °C remained undamaged during the whole storage period and did not show any symptoms of CI. Symptoms were characterized by brown depressed pitting areas that progressively became dark-brown lesions covering larger surface of the fruits as advanced the cold storage period (Figure 1(a)). CI incidence increased progressively during the storage, reaching values around 2 in MSH and 1.5 in SR variety after two months, and it did not increase substantially after the simulation of the shelf-life period. A pre-treatment with the ethylene action inhibitor, 1-MCP, reduced CI damage in SR fruit, which was less prone to develop CI. However, this effect of 1-MCP was not corroborated in MSH variety (Figure 1) and could not be consistently reproduced in fruits harvested in different seasons (data not shown). It is interesting to remark that in fruits of the red SR grapefruit, CI symptoms were restricted only to the yellow areas of the peel, and those of red coloration were virtually free of pitting or any other CI symptoms (data not shown). Many of the fruits of SR grapefruit developed red areas in the peel although of variable extension and intensity, and this may be the reason of the lower CI index in fruits of this grapefruit in comparison with those of MSH (Figure 1(a) and (b)). This tolerance of red-colored areas of the peel of SR grapefruit to CI has been the subject of parallel studies and investigations (Lado et al., unpublished results).

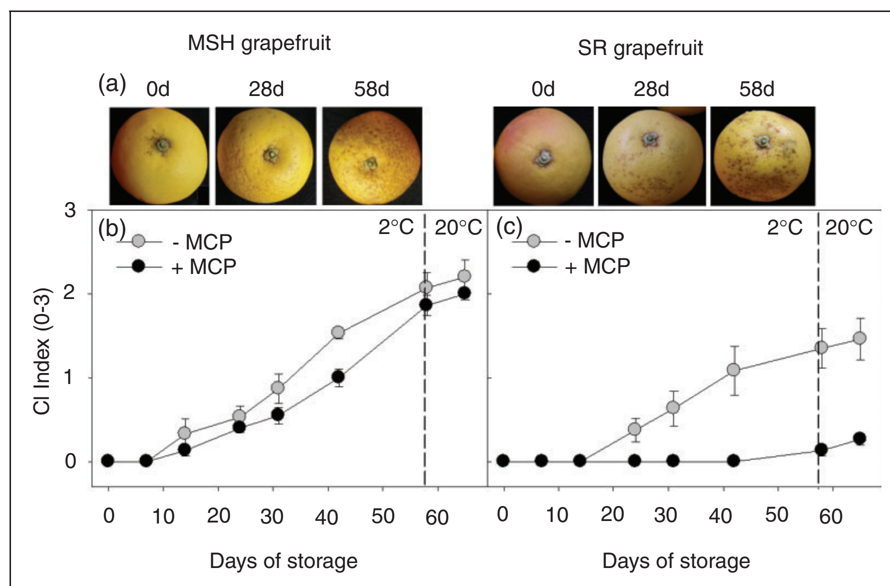

Figure 1. (a) Chilling injury symptoms in the peel of MSH and SR grapefruit after 28 and 58 days of storage at 2 °C and (b) evolution of CI (0–3) during storage at 2 °C for up to 58 days and after seven days of shelf-life at 20 °C in fruits of MSH, and (c) SR grapefruits treated or non-treated with 1-MCP.

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Ethylene production was very low at the harvest time in fruits of both varieties and experienced only minor changes during storage of non-treated control fruit (Figure 2). By contrast, inhibition of ethylene perception by 1-MCP markedly modified the rate of ethylene production in fruits of both varieties. After four weeks in MSH or two weeks storage in SR, 1-MCP-treated fruits produced more than 10 times ethylene compared with control fruits (inset panels Figure 2). This overstimulation of ethylene production by 1-MCP was also manifested after rewarming. Then, only one day after transfer from 2 °C to 20 °C, a massive burst of ethylene production was measured reaching values as higher as 12–15 nL/h/g (Figure 2). This massive increase on ethylene production in 1-MCP-treated fruit is consistent with previous results in citrus fruits (Katz et al., 2004; Lafuente et al., 2001; Marcos et al., 2005; Mullins et al., 2000) and reinforced the motion of a negative feedback regulation by ethylene of its own biosynthesis. This mechanism was initially described in the wound-induced ethylene production in the peel of citrus fruit (Riov and Yang, 1982) and has been also observed in pathogen and cold-induced ethylene biogenesis (Lafuente et al., 2001; Marcos et al., 2005). Autoinhibition of stress-induced ethylene production implicates that ethylene represses the biochemical and molecular processes controlling its own biosynthesis and under absence of ethylene action, as by 1-MCP treatment, the inhibition is lost and ethylene production is over-stimulated (El-Otmani et al., 2011), as was observed in cold-stored grapefruits (Figure 2).

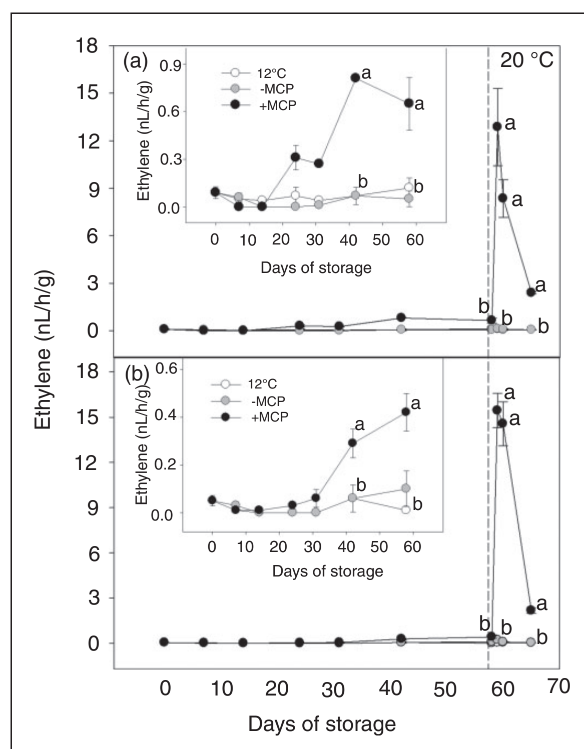

Figure 2. Ethylene production (nL/h/g) in (a) MSH and (b) SR grapefruits treated or non-treated with 1-MCP and stored for up to 58 days at 2 °C and after seven days of shelf-life at 20 °C. Control fruit was stored at 12 °C and transferred to 20 °C for shelf-life simulation. Inserted panel shows ethylene production in MCP-treated and non-treated fruits stored at 2 °C and 12 °C for up to 58 days. Different letters indicate significant differences between treatments at each date ($p \leq 0.05$).

Then, a positive relation between damage and ethylene emission could be suggested, as observed previously in cold-stored Fortune mandarin (Zacarías et al., 2003). The involvement of ethylene in the development of CI is controversial and depends on the plant species since ethylene may play a preventive (Dong et al., 2001; Zhou et al., 2001) or inductive role (Dou et al., 2005b; Edagi et al., 2010; Orihuel-Iranzo et al., 2010). In citrus fruit, there is also controversial the involvement of ethylene in CI development and if it is a cause or a consequence of chilling damage is still unclear. In Marsh grapefruits and Fortune mandarin, the chilling-induced ethylene production was coincident with the initiation of CI symptoms (McCollum and McDonald, 1991) and ethylene not always is able to mitigate the chilling-induced damage (Gosalbes et al., 2004; Lafuente et al., 2001; Porat et al., 1999). Moreover, experiments with 1-MCP did not show consistent results on CI development in fruits of different citrus varieties, and protective (Salvador et al., 2006) and deleterious effects (Lafuente et al., 2001) have been reported. Under other stress conditions, as pathogen infection, it has been observed that disruption of ethylene perception enhanced deleterious effects, suggesting that ethylene may be part of the protective role of the fruits to cope stress conditions (Marcos et al., 2005; Porat et al., 1999). The discrepancy on the relationship between ethylene and cold stress observed among fruits of different citrus varieties, suggests that the response may be dependent on the variety and also on the particular sensitivity to CI, among other factors (Watkins, 2006).

To further understand the regulation of ethylene biosynthesis and perception during grapefruits cold storage, the expression of ethylene biosynthesis (ACC synthase-*ACS1* and *ACS2* and ACC oxidase-*ACO*), receptors (*ETR1*, *ETR2*, and *ETR3*), and response factors (*ERF1* and *ERF2*) genes was analyzed in the peel of fruits stored at 2 °C and after shelf-life at 20 °C. The results of transcriptional analysis of these genes were virtually the same in both grapefruit varieties, indicating a similar regulation of ethylene metabolism, and therefore, and to avoid repetitive data, only results on

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Marsh genotype are thoroughly described (Figures 3 to 5).

Cold storage markedly induced the expression of the ethylene biosynthetic genes *ACS1*, *ACS2,* and *ACO* which was especially overstimulated in the peel of 1-MCP pre-treated fruit, showing that the negative feedback regulation of the cold-induced ethylene production acts at transcriptional level (Figure 3). It is interesting to mention that although both *ACS1* and *ACS2* transcripts were cold induced, the increase of the former was higher. In fruits of other citrus varieties exposed to different stressful conditions, differential accumulation of both *ACS* transcripts was observed, indicating a tissue or stress-specific response (Katz et al., 2004; Marcos et al., 2005; Zacarías et al., 2003). Despite the transcription of *ACS* and *ACO* genes was also induced in response to cold (although with a different extent), this increase was not directly reflected on ethylene production during cold storage (Figure 2). This effect could be explained by the partial inactivation of ACO enzyme activity under low temperatures (Lederman et al., 1997), a limitation that was clearly removed during shelf-life at 20 °C (Figure 2). Thus, after fruit rewarming the enzymes of ethylene production may reach a temperature near optimum for activity, and therefore, ethylene production would be triggered at expenses of the metabolites (ACC) accumulated during the exposition to cold stress, similar to that observed in other citrus cultivars (Zacarías et al., 2003). Moreover, after stress removal, *ACS1* and *ACO* genes expression declined (Figure 3), indicating that cold directly triggers transcription of the ethylene biosynthetic genes. A similar induction of *ACS* and *ACO* genes during cold storage has been also

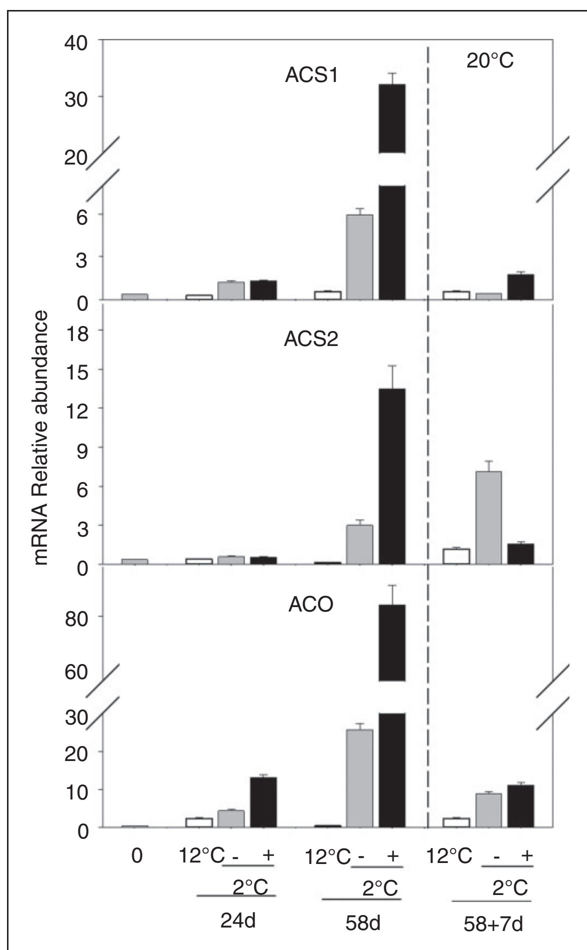

Figure 3. Expression of ethylene biosynthetic genes *ACC synthase-ACS1* and *ACS2* and *ACC oxidase-ACO* in the peel of MSH grapefruit treated (+) and non-treated (–) with 1-MCP and stored for 24 and 58 days at 2 °C and after seven days of shelf-life at 20 °C. Control fruit was stored at 12 °C and transferred to 20 °C for shelf-life simulation.

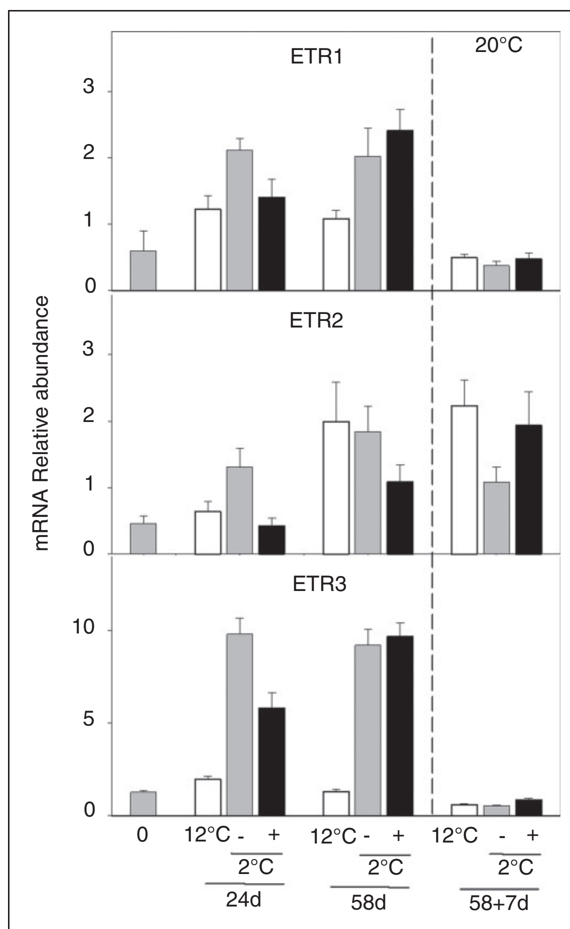

Figure 4. Expression of ethylene receptor genes *ETR1, ETR2,* and *ETR3* in the peel of MSH grapefruit treated (+) and non-treated (–) with 1-MCP and stored for 24 and 58 days at 2 °C and after seven days of shelf-life at 20 °C. Control fruit was stored at 12 °C and transferred to 20 °C for shelf-life simulation.

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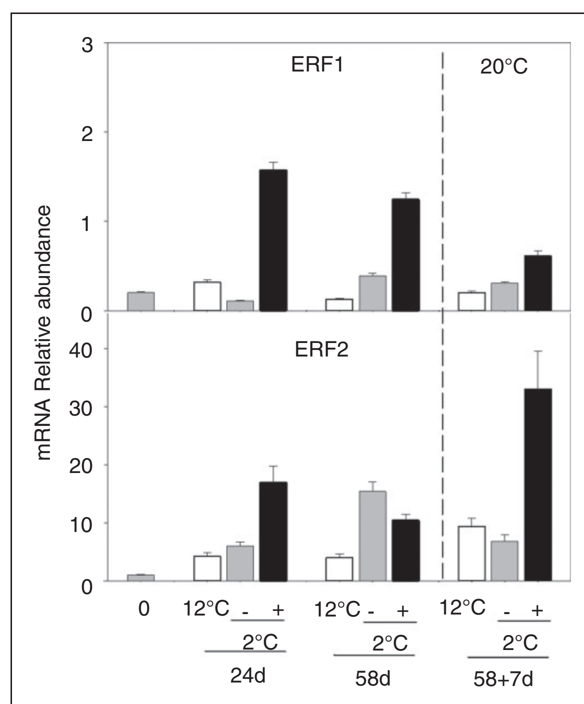

Figure 5. Expression of ethylene response factors genes *ERF1* and *ERF2* in the peel of MSH grapefruit treated (+) and non-treated (–) with 1-MCP and stored for 24 and 58 days at 2 °C and after seven days of shelf-life at 20 °C. Control fruit was stored at 12 °C and transferred to 20 °C for shelf-life simulation.

described in MSH grapefruit stored under cold conditions (Maul et al., 2008; Maul et al., 2011). *ACS1* and *ACS2* genes were also stimulated in oranges stored at 4 °C and drastically declined after fruit removal to 25 °C, coinciding with a marked increase on ethylene production at higher temperature (Wong et al., 1999). It is worth mentioning that the stimulation of *ACS1*, *ACS2,* and *ACO* transcripts by 1-MCP is in agreement with the increase of ethylene production in 1-MCP-treated fruits exposed to 2 °C. Although this stimulation of ethylene production was significant with respect to non-1-MCP-treated fruits, it was much lower to the increase registered after rewarming, that reached ethylene production levels as high as 15 nL/h/g (Figure 2). Interestingly, after transference to 20 °C, *ACS* and *ACO* transcripts abundance declined, indicating that cold is likely the stimuli inducing the expression of these genes and that ethylene is a negative regulator of its own biosynthesis in grapefruits.

To analyze the effect of cold or chilling in the regulation of ethylene perception and signaling, the expression of three members of the *ETR* gene family was measured during cold storage and shelf-life. The results of Figure 4 indicated that each *ETR* gene member analyzed was differentially and independently induced by cold and/or ethylene. The three *ETR* genes were early up-regulated by cold, especially *ETR3*, since its relative expression level was higher in cold-stored fruits compared to that in fruits exposed to non-chilling temperatures. The stimulation of *ETR1* and *ETR3* expression by low temperature was maintained after 58 days, but this did not occur with *ETR2*. Inhibition of ethylene action by 1-MCP produced variable effects on the expression of the three *ETR* genes since it reduced the cold-induced expression after 24 days but not after a more prolonged cold storage (Figure 4). After fruit rewarming, expression of both *ETR1* and *ETR3* genes was substantially reduced, whereas that of *ETR2* remained with minor modifications. Collectively, these results indicated that *ETR3* gene was the most responsive to variations in storage temperature and that this gene and *ETR1* appear to be cold-induced. The involvement of ethylene in the regulation of *ETR* genes is complex with an apparent repression of the cold-induced stimulation in grapefruits (Figure 4). Ethylene-signaling cascade is composed of a interrelated network of different regulatory steps (Gapper et al., 2013; Wang et al., 2013) where the first responsible mechanism for the sensing of the hormone are ETRs that are a complex family of isoforms varying in number in the different species. Five isoforms of receptors have been described in *Arabidopsis* while six different elements were reported to exist in tomato fruit (Binder, 2008). In citrus fruit, three different isoforms for the ETR family of ETRs have been described (Distefano et al., 2009; John-Karuppiah and Burns, 2010; Katz et al., 2004) and some of them, mainly *ETR2,* was induced by exogenous ethylene application during color change (Alós et al., 2014a). Moreover, a positive correlation between transcript levels of *ETR*s and ethylene sensitivity was suggested in citrus (Alós et al., 2014a) and in apple fruit (Yang et al., 2013). These changes in the expression of *ETR*s may be relevant to determine tissue sensitivity to the hormone, since the ethylene action is thought to be negatively regulated by ETRs (Gapper et al., 2013) and a high receptors level would repress ethylene response while low amount would enhance ethylene sensitivity (Agarwal et al., 2012). However, this process is very complex and experimental evidences are not always correlated with the predicted model. In apple fruits, for example, the level of ETR genes increased with stimulation of ethylene response and natural ripening (Tatsuki et al., 2009; Yang et al., 2013). Our results are the first evidences of the involvement of the *ETR* gene family of ETRs in the citrus fruits response to cold storage.

Following downstream elements in the ethylene signaling cascade, ethylene response factors (*ERF*s) have been described as negative regulators of ethylene responses and essential modulators of fruit ripening

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and stress responses (Gapper et al., 2013; Wang et al., 2013). *ERF*s exhibited variable regulation by cold and 1-MCP treatments in grapefruits. The expression of *ERF1* was markedly stimulated by 1-MCP but not by low temperature (Figure 5). On the other hand, *ERF2* transcription was stimulated by cold and 1-MCP treatment both during low temperature storage and shelf-life at 20 °C (Figure 5). These results suggest that these two ERF factors, especially *ERF2,* may be involved in the cascade of events induced during cold stress in grapefruits and that are negatively regulated by ethylene, since the 1-MCP overstimulated their expression (Figure 5). Similarly, it has been described that both *ERF*s are negatively regulated by ethylene in the peel of Comune mandarin (Alós et al., 2014a) while another group of ERFs (C-repeated binding factor-CBFs) have been described to be induced under cold and to display a relevant role in cold hardiness in citrus fruit and the close-related *Poncirus* (Champ et al., 2007).

CONCLUSIONS

Collectively, from the results of the current study, it could be concluded that cold markedly stimulates transcription of ethylene biosynthetic genes (*ACS* and *ACO* genes) and to a lesser extent, transcript abundance of a specific isoform of the ethylene receptor *ETR3*, and also of the ethylene response factor *ERF2*. Pre-treatment of grapefruit with the inhibitor of ethylene action, 1-MCP, before storage at 2 °C induced a significant increase in ethylene production during cold storage that became massive after simulation of shelf-life at 20 °C, indicating a negative feedback control by ethylene of its own biosynthesis. No clear effect of 1-MCP was observed in the induction of *ETR* gene expression. However, ethylene appears to be implicated in the transcriptional regulation of *ERFs* both under cold storage and during shelf-life.

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Fruit shading enhances peel color, carotenes accumulation and chromoplast differentiation in red grapefruit

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The distinctive color of red grapefruits is due to lycopene, an unusual carotene in citrus. It has been observed that red 'Star Ruby' (SR) grapefruits grown inside the tree canopy develop a more intense red coloration than those exposed to higher light intensities. To investigate the effect of light on SR peel pigmentation, fruit were bagged or exposed to normal photoperiodic conditions, and changes in carotenoids, expression of carotenoid biosynthetic genes and plastid ultrastructure in the peel were analyzed. Light avoidance accelerated chlorophyll breakdown and induced carotenoid accumulation, rendering fruits with an intense coloration. Remarkably, lycopene levels in the peel of shaded fruits were 49-fold higher than in light-exposed fruit while concentrations of downstream metabolites were notably reduced, suggesting a bottleneck at the lycopene cyclization in the biosynthetic pathway. Paradoxically, this increment in carotenoids in covered fruit was not mirrored by changes in mRNA levels of carotenogenic genes, which were mostly up-regulated by light. In addition, covered fruits experienced profound changes in chromoplast differentiation, and the relative expression of genes related to chromoplast development was enhanced. Ultrastructural analysis of plastids revealed an acceleration of chloroplasts to chromoplast transition in the peel of covered fruits concomitantly with development of lycopene crystals and plastoglobuli. In this sense, an accelerated differentiation of chromoplasts may provide biosynthetic capacity and a sink for carotenoids without involving major changes in transcript levels of carotenogenic genes. Light signals seem to regulate carotenoid accumulation at the molecular and structural level by influencing both biosynthetic capacity and sink strength.

Abbreviations – βCHX, β-carotene hydroxylase; βLCY, lycopene cyclase β; ABA, abscisic acid; C, covered; Chl, chlorophyll; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; FIB, fibrillin; FW, fresh weight; GGPP, geranyl geranyl pyrophosphate; GGPPS, geranyl geranyl pyrophosphate synthase; HDR, hydroxymethylbutenyl diphosphate reductase; HPLC, high-performance liquid chromatography; MEP, methyl-D-erythritol-4-phosphate; NC, non-covered; PCR, polymerase chain reaction; PDS, phytoene desaturase; PSY, phytoene synthase; sHSP, small heat shock protein; SR, Star Ruby; ZDS, ζ-carotene desaturase.

Introduction

Carotenoids are isoprenoid-derived pigments accumulated in the peel and pulp of citrus fruit and are responsible for the high diversity of colorations within this crop (Gross 1987). During the last decade, considerable effort has been directed at trying to understand how color and carotenoid composition are regulated in citrus fruits, identifying key genes of the pathway and key metabolic steps (Kato et al. 2004, Rodrigo et al. 2004, 2013, Alquézar et al. 2008, Kato 2012). Mature oranges preferably accumulate xanthophylls, mainly 9-*cis*-violaxanthin (up to 80% of total carotenoids), which are responsible for their typical pale orange-yellowish color, while β-cryptoxanthin and the apocarotenoid β-citraurin are also present in mandarin fruit, contributing to the intense orange coloration displayed by some varieties (Rodrigo et al. 2013). On the other hand, mature white grapefruits accumulate minute amounts of carotenoids, mainly phytoene and violaxanthin, while red grapefruits are able to accumulate large amounts of linear carotenes, mainly phytoene, phytofluene and the red lycopene in both peel and pulp (Alquézar et al. 2013). Furthermore, levels of carotenoids are similar in the peel and the pulp of red grapefruits, contrary to the common feature in other citrus fruits that display much higher carotenoid content in the peel than in the pulp (Alquézar et al. 2013, Rodrigo et al. 2013).

The peel of immature citrus fruit accumulates mainly lutein and minor proportions of other chloroplastic carotenoids (zeaxanthin, β- and α-carotene). At the onset of fruit coloration, lutein declines and almost disappears in parallel to a marked increase of colored carotenoids, mostly β,β-xanthophylls (Kato et al. 2004, Rodrigo et al. 2004). These changes are transcriptionally coordinated with a parallel up-regulation of genes of the carotenoid biosynthetic pathway (Fig. 1). The process is initiated by the up-regulation of the phytoene synthase (*PSY*) (the first committed step of the pathway) and followed by an increment in the expression of phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS*), β-carotene hydroxylase (β-*CHX*) and the induction of the chromoplast-specific lycopene cyclase 2 (β*LCY2*) genes (Kato et al. 2004, Rodrigo et al. 2004, Alquézar et al. 2008, 2009, 2013).

Accumulation of lycopene in citrus fruit is of particular interest because it is an unusual feature restricted to only a few species such as grapefruit (*Citrus paradisi*), pummelo (*Citrus grandis*) and the flesh of some orange (*Citrus sinensis*) mutants (Liu et al. 2007, Alquézar et al. 2008, 2013). Recent studies indicate that differential mechanisms may be operating in lycopene-accumulating mutants (Alquézar et al. 2009, Pan et al. 2009, Xu et al.

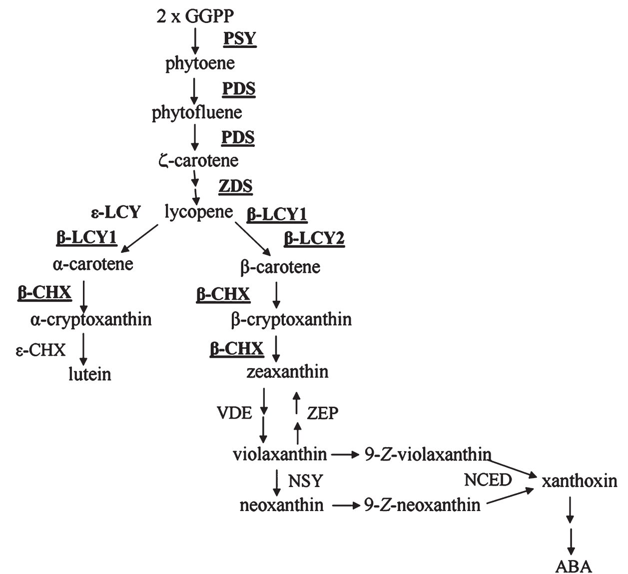

Fig. 1. Schematic diagram of the biosynthesis pathway of carotenoids in citrus fruit. Genes underlined were analyzed in this study.

2010, Mendes et al. 2011, Costa et al. 2012). In the red 'Hong Anliu' orange, a higher expression of PSY and ZDS genes and lower of βLCYs compared to the parental variety appears to be a key factor triggering lycopene accumulation (Xu et al. 2010). Similarly, in the red grapefruits 'Star Ruby' (SR) and 'Flame', the presence of lycopene seems to be related to a lower expression level of the fruit-specific βLCY2 gene (Alquézar et al. 2009, 2013, Mendes et al. 2011) while in Cara Cara orange it has been associated with an enhanced expression of the genes of the methyl-D-erythritol-4-phosphate (MEP) pathway (Alquézar et al. 2008), the main route supplying precursors for carotenoid biosynthesis. The flux into the MEP pathway is controlled by two key enzymes: the 1-deoxy-D-xylulose-5-phosphate synthase (DXS), located upstream, and the hydroxymethylbutenyl diphosphate reductase (HDR), downstream in the pathway, constituting two regulatory steps that directly influence carotenoid accumulation (Botella-Pavía et al. 2004, Peng et al. 2013). At the end of the MEP pathway, the formation of the key precursor geranyl geranyl pyrophosphate (GGPP), which constitutes a key branch point in isoprenoid biosynthesis in plants, is catalyzed by the enzymes GGPP synthases (GGPPS), a complex family of different isoforms that vary in expression, localization and activity (Beck et al. 2013). Most GGPP synthases have been shown to be localized in plastids and are directly related to carotenoid accumulation in vitro (Thabet et al. 2012). Their gene transcript levels and enzymatic activity were strongly induced during color change in ripening pepper fruits (Kuntz et al. 1992).

Carotenoid synthesis takes place inside plastids (chloroplasts and chromoplasts) (Gross 1987), and the differentiation from chloroplast to chromoplasts is concomitant with massive carotenoid accumulation during fruit ripening (Gross 1987, Cazzonelli and Pogson 2010). The amount of carotenoids accumulated in plastids is determined by both the equilibrium between biosynthesis and degradation and the capacity to develop sink structures (Cazzonelli and Pogson 2010). Chromoplasts develop diverse structures for carotenoid storage, varying in shape and size, depending on the fruit and its developmental stage (Sitte et al. 1980, Gross 1987). The predominant structures present inside chromoplasts are related to the type of carotenoid accumulated (Wang et al. 2013). The most common structure for carotenoid accumulation inside citrus peel chromoplasts is the spherical plastoglobuli which increase in abundance as fruit ripen (Gross 1987, Cao et al. 2012). In the peel of 'Valencia' oranges and 'Goliath' pummelo chromoplasts, a combination of plastoglobuli and well-organized parallel achlorphyllous membranes have been observed (Gross 1987). On the other hand, crystals were proposed as the predominant structures for lycopene accumulation in tomato fruit (Simkin et al. 2007, Schweiggert et al. 2011, Nogueira et al. 2013) and were also present in lycopene-accumulating grapefruits (Cao et al. 2012).

Different proteins, mainly chaperones, are involved in the ultrastructural changes that take place during chromoplast differentiation. Among them are the fibrillins or plastoglobulins (FIBs or PAPs), the orange (Or) protein and small heat shock proteins (sHSPs). FIBs are the predominant proteins inside the plastoglobuli and are thought to assist in the globular-fibrillar transition which allows for the storage of high amounts of newly synthetized carotenoids during chloroplasts to chromoplast conversion (Simkin et al. 2007). An increased accumulation of *FIBs* (*CitPAP*) transcripts was described in citrus fruit during the color change (Moriguchi et al. 1998). Similarly, the overexpression of *Or* gene in sweet potato led to an increase in carotenoids, mainly β-carotene and lutein (Kim et al. 2013), while overexpression of the *Or* gene from *Arabidopsis thaliana* in rice callus caused a massive carotenoid accumulation (Bai et al. 2014). Other proteins involved in chromoplast differentiation are the sHSPs, which are thought to be directly related to fruit ripening (Neta-Sharir et al. 2005, Pan et al. 2012). They are abundant in orange chromoplasts (Zeng et al. 2011) and differentially expressed among citrus varieties (Pan et al. 2012).

Light, both intensity and quality, is one of the most crucial environmental factors influencing carotenoid accumulation in plant tissues (reviewed in Pizarro and Stange 2009). It has been shown that the MEP pathway and *PSY* transcription are stimulated by light (Botella-Pavía et al. 2004, Toledo-Ortiz et al. 2010). Moreover, light induced carotenoid biosynthetic genes in photosynthetic tissues (Simkin et al. 2003), in addition to enhance carotenoid accumulation in tomato fruit (Schofield and Paliyath 2005, Azari et al. 2010). In contrast, darkness is needed for the burst in gene expression and carotenoid accumulation in carrot roots (Fuentes et al. 2012, Rodriguez-Concepcion and Stange 2013). Little information is available about the effect of light on carotenoid biosynthesis in citrus fruit. In citrus species, such as sweet oranges and mandarins, light exposure during fruit ripening generally enhances carotenoid accumulation and external fruit color (Cronje et al. 2011, 2013). In contrast, SR grapefruit develop more intense red peel coloration when grown under low light conditions (field observations), indicating a different response to light levels compared to mandarins and oranges. To understand further the regulatory mechanisms of light on carotenoids biosynthesis in the lycopene-accumulating red grapefruit 'Star Ruby', carotenoid composition and expression of carotenoid biosynthetic genes were analyzed in the peel of shaded fruit in comparison to those exposed to light under standard photoperiodic conditions. Additionally, abscisic acid (ABA) content, a downstream metabolite of the carotenoid pathway, was also analyzed. We further explored possible changes in the structure of plastids during chloroplasts to chromoplast conversion, as well followed the expression of genes related to the transition of chloroplasts to chromoplasts such as those encoding enzymes like FIBs, Or and sHSPs.

Materials and methods

Plant material and treatments

Complete branches located inside and outside of the canopy of *C. paradisi* cv. 'Star Ruby' trees were tagged and color changes during ripening followed in orchards located in the Northern (39°37′20′′N, 0°33′38′′W) and Southern (28°78′69′′S, 20°65′19′′E) hemispheres by using a Minolta CR-330 colorimeter (Minolta, Osaka, Japan). The orchard in the Northern hemisphere was located in Lliria (Valencia, Spain) with an average of 2660 h of sunlight per year (heliophany) and a rainfall level of 454 mm (data from 1971 to 2000), and the orchard in Southern hemisphere was located in Kakamas with 3731 h of heliophany and precipitation of 174.3 mm per year. For covering experiments, 105 fruits located outside of the canopy from four different trees of *C. paradisi* cv. 'Star Ruby' growing under commercial

conditions in Lliria, Valencia (Spain) were tagged. Half of the tagged fruits were covered with black plastic bags in July at an immature green stage (55 ± 1 mm fruit diameter), before the initiation of natural degreening process, coinciding with 118 DAA (days after anthesis), and the bag end-bottom was left open to allow gas exchange. Three biological replicate samples of five fruit each were harvested as a starting point. In October, November and December, coinciding with mature green (187 DAA), breaker (224 DAA) and full color (257 DAA) stages, three 5-fruit replicates for control fruit (non-covered, NC) and covered fruit (C) were harvested and the peel tissue excised, frozen in liquid nitrogen, ground to a fine powder and stored at −80°C until further analyses. At each harvest date, peel color of whole fruit was measured using a Minolta CR-330 colorimeter (Minolta, Osaka, Japan) on three areas of the equatorial plane of the fruit and expressed as the *a/b* Hunter ratio (Stewart and Wheaton 1972). The *a/b* ratio is negative for green fruit, the zero value corresponds to yellow fruit at color break and orange to red colored fruit reflects a positive value.

Chlorophyll and carotenoid extraction

Peel pigments were extracted as previously described (Alquézar et al. 2008). The chlorophyll (Chl) (a + b) content was determined by measuring the absorbance at 644 and 662 nm and calculated according to the Smith and Benitez (1955) equations. After Chl measurements, the pigment ethereal solution was dried and saponified using a 10% methanolic:KOH solution. Carotenoids were extracted and the samples dried under N_2 and kept at −20°C until analysis. All procedures were carried out on ice under dim light to prevent possible photodegradation, isomerization and structural changes of carotenoids.

Carotenoid analysis by HPLC

Carotenoid composition of each sample was analyzed by high-performance liquid chromatography (HPLC) with a Waters liquid chromatography system equipped with a 600E pump, a model 996 photodiode array detector, and Empower software (Waters, Barcelona, Spain). A C30 carotenoid column (250 × 4.6 mm, 5 μm) coupled to a C30 guard column (20 × 4.0 mm, 5 μm) (YMC Europe GmbH, Schermbeck, Germany) was used. Samples were prepared for HPLC by dissolving the dried carotenoid extracts in $CHCl_3$:MeOH:acetone (3:2:1, v:v:v). A ternary gradient elution with MeOH, water and methyl *tert*-butyl ether (MTBE) was used for carotenoid separation as reported in previous work (Alquézar et al. 2008). The carotenoid peaks were integrated at their individual maxima wavelength, and their content was calculated using the following calibration curves: β-cryptoxanthin (Extrasynthese, Genay, France) for α- and β-cryptoxanthin; lutein (Sigma, Steinheim, Germany) for lutein, neoxanthin and violaxanthin isomers; lycopene (Sigma, Steinheim, Germany); zeaxanthin (Extrasynthese, Genay, France) for zeaxanthin and antheraxanthin; α- and β-carotene (Sigma, Steinheim, Germany). Standards for calibration curves of phytoene, phytofluene and ζ-carotene were obtained from flavedo extracts of 'Pinalate' fruits, which accumulate large amounts of these compounds. Samples were extracted twice, and each analytical determination was replicated at least twice. All operations were carried out on ice under dim light to prevent photodegradation, isomerization and structural changes of carotenoids. To test for significant differences in carotenoids concentration between NC and C fruit at each harvest date, a two-tailed unpaired Student's *t*-test was applied ($P \leq 0.05$).

Quantitative real-time PCR

Total RNA was isolated from the flavedo of fruit at each harvest date, using RNeasy Plant Mini Kit (Qiagen, Madrid, Spain) and subsequently treated with DNase (DNA free, DNase treatment and removal, Ambion, Madrid, Spain). The transcripts present in 2 μg of total RNA were reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen, Madrid, Spain) in a total volume of 20 μl. One microliter of a five times diluted first-strand cDNA, containing approximately 100 ng of cDNA, was used for each amplification reaction. Quantitative real-time polymerase chain reaction (PCR) was performed on a LightCycler 480 instrument (Roche, Madrid, Spain), using the LightCycler 480 SYBR-Green I Master kit (Roche, Madrid, Spain). Reaction mix and conditions followed the manufacturer's instructions. The primers employed for the amplification of each gene are described in Table S1 (Supporting Information). The protocol for all the genes analyzed consisted of 10 min at 95°C for pre-incubation, followed by 40 cycles of 10 s at 95°C for denaturation, 10 s at 59°C for annealing and 10 s at 72°C for extension. Fluorescent intensity data were acquired during the extension time. Specificity of the PCR reaction was assessed by the presence of a single peak in the dissociation curve performed after the amplification steps. For expression measurements, LightCycler 480 Software release 1.5.0, version 1.5.0.39 (Roche, Madrid, Spain), was used, and expression levels relative to values of a reference sample were calculated using the Relative Expression Software Tool (REST; Pfaffl et al. 2002). Normalization was performed using the expression levels of the *actin* gene based on previous

housekeeping selection (Alós et al. 2014). For all genes analyzed, the reference sample was the expression value obtained in the peel of fruit harvested in July which was set at one. Results were expressed as the average of four independent replicates. To test for significant differences on transcript levels between NC and C fruit at each harvest date, a pair-wise fixed reallocation randomization test was applied ($P \leq 0.05$).

ABA quantification

Analysis was carried out by HPLC coupled to tandem mass spectrometry as described in de Ollas et al. (2013). Briefly, 0.4 g of frozen fruit peel was extracted in 5 ml of distilled water after spiking with 100 ng of [$^2H_6$]-ABA in order to avoid matrix effects and to assess recovery. After centrifugation at 4°C, supernatants were recovered and pH adjusted to 3.0 with 30% acetic acid. The acidified water extract was partitioned twice against 3 ml of diethyl ether. The organic layers were combined and evaporated under vacuum. The dry residue was then re-suspended in a 10% MeOH solution by gentle sonication. The resulting solution was filtered and directly injected into a HPLC system (Waters Alliance 2695, Milford, MA, USA). ABA was separated in a reversed-phase Kromasil 100 C18 column (100 × 2.1 mm, 5 μm particle size; Scharlab, Barcelona, Spain) using a linear gradient of MeOH and water supplemented with 0.01% acetic acid at a flow rate of 300 μl min^{-1}. Quantification was performed with triple quadrupole mass spectrometer (Quattro LC, Micromass Ltd., Manchester, UK) connected online to the output of the column through an orthogonal Z-spray electrospray ion source. To test for significant differences between NC and C fruit at each harvest date, a two-tailed unpaired Student's *t*-test was applied ($P \leq 0.05$).

Transmission electron microscopy

The peel tissue samples were manually cut in small squares of 5 mm long × 1 mm wide, fixed in modified Karnovsky fixative (0.5% glutaraldehyde, 2.5% formaldehyde in 0.1 *M* phosphate buffer, pH 7.4). Specimens were then rinsed in 0.1 *M* piperazine-N,N′-bis (2-ethanesulfonic acid)-PIPES buffer, postfixed in 1% buffered osmium tetroxide (1 h), rinsed in buffer, block stained in 2% aqueous uranyl acetate (20 min), dehydrated in an ethanol series and embedded in Spurr resin (Elektron Technology, Stansted, UK) in the normal way. Gold/silver ultrathin sections were then cut from suitable regions of the tissue and stained with Reynolds lead stain and viewed on a Hitachi H7000 transmission electron microscope (Hitachi, Krefeld, Germany) equipped with a SIS Megaview III digital camera (Olympus, Southend-on-Sea, UK).

Results

Fruit position on the tree canopy affects peel color during ripening of SR grapefruit

In red lycopene-accumulating grapefruits, it was observed that the surface of the fruit covered by leaves or in contact with other fruit displayed a faster rate of degreening and also developed a more intense red coloration in the peel compared with those directly exposed to sunlight (Fig. S1). Based on these field observations, we first analyzed the effect of natural fruit shading on peel coloration of SR grapefruits by comparing peel color of fruits located on the outside of the tree canopy with those located inside and covered by leaves, from orchards of two distant locations in Northern (Lliria, Valencia, Spain,) and Southern (Kakamas, South Africa) hemispheres. Although the rate of fruit coloration was different in each location, probably due to differences in environmental and/or agronomic conditions, we consistently found that the red peel color (higher *a/b* ratio) was more intense in fruits growing inside the canopy than on the outside of the tree (Fig. 2).

Effect of fruit bagging on peel color, pigments composition and ABA content in SR grapefruit

To study the effect of light avoidance on color development and pigment composition in SR grapefruit, immature green fruits (July) growing outside the tree canopy were covered by black plastic bags (C) and compared with NC fruit, exposed to normal photoperiodic conditions. Changes in peel color and pigment concentration were determined periodically in both NC and C fruits throughout the ripening process, corresponding to mature green (October), breaker (November) and mature (December) stages for SR grapefruit (Alquézar et al. 2013). Covering SR fruit substantially accelerated peel color development, resulting in significant differences in the *a/b* Hunter color index compared to control fruit (Fig. 3). Three months after bagging (October), the peel of C fruit showed pink tones and reached an *a/b* ratio of 0.2 units, while in NC fruit the ratio was less than −0.2, corresponding to green-yellowish coloration. Differences in color development between both treatments were maintained until the end of the ripening period, when mature C fruits were fully red colored (*a/b* ratio of 0.45) whereas NC fruits were slightly yellow-orange with an *a/b* ratio of one-tenth (*a/b* = 0.04; Fig. 3A).

In accordance with the rapid disappearance of green color in C fruits, a faster Chl breakdown occurred. In the peel of NC fruit harvested in October, Chl content decreased by 27% whereas those covered fruit declined by 83%. In November, Chl concentration in the peel

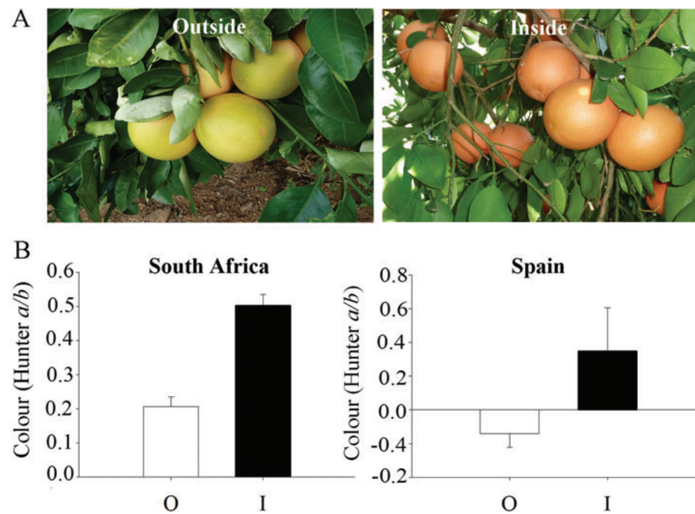

Fig. 2. Effect of fruit shading on peel color development in Star Ruby (SR) fruit. (A) Pictures representative of the external color of SR fruit developed inside or outside of the tree canopy. (B) Color (*a/b* Hunter) in fruit developed inside (I) or outside (O) of the canopy harvested in May (270 days after anthesis) in South Africa and in November (260 days after anthesis) in Spain.

of NC fruits was still 33% of the initial values, but in C fruit they were less than 10% of the original values. No Chls were detected in the peel of both NC and C fruit in December (Fig. 3B). Total carotenoid content in the peel of SR fruit at bagging time was 18.9 $\mu g\,g^{-1}$ fresh weight (FW) and remained almost constant until December in fruit exposed to normal photoperiodic conditions. In contrast, total carotenoid levels increased nearly four fold in the peel of C fruit in October to reach concentrations near 80 $\mu g\,g^{-1}$ FW in November and December (Fig. 3C).

The effect of fruit bagging on the concentration of specific carotenoids was also determined (Table 1). The peel of immature green fruits (July) showed a carotenoid profile typical of chloroplastic tissue, lutein being the predominant carotenoid, followed by violaxanthin, β-carotene and neoxanthin, while linear carotenes represented only 6% of total carotenoids (Table 1). Although total carotenoid content experienced minor changes in NC fruit during ripening, individual carotenoids levels showed important modifications. Phytoene increased from 6 and 12 times in NC fruits in October and December, respectively, but it was always half of the content found in C fruits. Thus, linear carotenoids accounted for 37 and 75% of total carotenoids in NC fruits in the period from October to December (Table 1). Lutein, violaxanthin and neoxanthin contents decreased markedly during ripening (Table 1). β-carotene content in NC fruit remained almost constant until November, dropping to less than 1.0 $\mu g\,g^{-1}$ FW in mature fruit (Table 1). Contrastingly, the peel of C fruit showed a massive accumulation of phytoene and phytofluene reaching values around 22 and 6 $\mu g\,g^{-1}$ FW in December, respectively, when linear carotenes represented more than 90% of total carotenoids in the peel of C fruits (Table 1). The decline in lutein and other xanthophylls was faster in C fruits than in NC during ripening (Table 1). It is worth noting that the most striking difference in carotenoids composition between NC and C fruit was the drastic increase of lycopene in the peel of the latter. Lycopene concentration at time of bagging was almost negligible and increased to 35 and 45 $\mu g\,g^{-1}$ FW in October and December, respectively, in C fruit (Table 1). Lycopene content at the same harvest dates in NC fruits was, respectively, 1/30th and 1/49th of that in C ones (Table 1).

To investigate whether the effect of light avoidance on carotenoid content may also affect the accumulation of ABA, a direct downstream product of the carotenoid pathway, its content was determined in the peel of NC and C fruit. ABA peel content progressively increased with ripening of NC fruit, reaching a maximum in November. Under dark conditions, the increment in ABA was delayed and reduced, being at the most half in C compared with NC fruits at all stages analyzed (Table 2).

Effect of fruit bagging on transcript accumulation of carotenoid biosynthetic genes in the peel of SR grapefruit

To analyze whether changes in carotenoid composition induced by light were related to alterations in the expression of key genes of carotenoid biosynthesis, the relative transcript abundance of genes *PSY*, *PDS*, *ZDS*, *βLCY1*, *βLCY2* (a and b alleles) and *βCHX*, covering

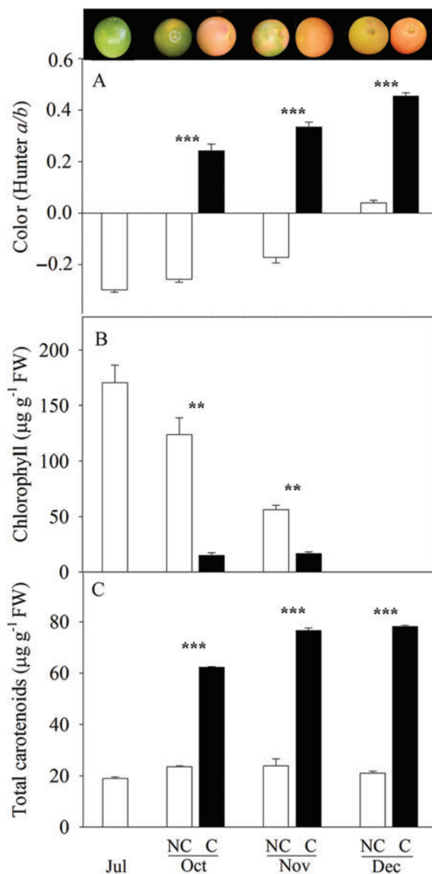

Fig. 3. Effect of fruit bagging on (A) peel coloration (*a/b* Hunter), (B) total chlorophyll content and (C) total carotenoid content (µg g^{-1} FW) in Star Ruby grapefruits. Immature green fruit were bagged in July, and non-covered (NC) and covered (C) fruit were harvested in October (69 days after bagging, dab), November (106 dab) or December (139 dab), corresponding to mature green, breaker and full color stages in NC fruit. Asterisks indicate significant differences between NC and C fruit at each harvest date (**$P \leq 0.01$, ***$P \leq 0.001$).

main steps of the pathway (Fig. 1), was analyzed in the peel of NC and C grapefruits. Transcript levels of most of the genes analyzed (*PSY*, *PDS*, *ZDS1*, *βLCY1* and *βCHX*) were enhanced during fruit ripening and were notably higher in light-grown fruits, reaching maximum levels in November and decreasing thereafter (Fig. 4). The accumulation of transcripts of both *βLCY2* alleles, described as key regulators of the conversion of lycopene to *β*-carotene in citrus fruits (Alquézar et al. 2009, 2013), was highly stimulated by light throughout the whole ripening period (Fig. 4). A remarkable feature for all the genes analyzed (except for the alleles 2 and 3 of *ZDS*) was the significant down-regulation in the peel of C fruits compared to NC (Fig. 4). Thus, the increase in the transcript levels in fruit from July to October was of 7 times for *PSY* and *ZDS1* and 3 times for *PDS* in light-exposed fruits, whereas in C fruits these enhancements were only double or lower. On the other hand, transcription of genes, the products of which are downstream lycopene, such as *βLCY1*, *βLCY2a*, *βLCY2b* and *βCHX*, was similar in NC and C fruits until October, when their relative transcript levels were again lower in C than in NC fruits (Fig. 4). *ZDS2* and *ZDS3* were the only genes whose expressions remained constant throughout the ripening process as well as between treatments, thereby suggesting that these two isoforms may not play a major role in the carotenoid changes taking place in grapefruit peel during ripening (Fig. 4).

Effect of fruit bagging on transcript accumulation of key genes of the MEP pathway in the peel of SR grapefruit

We further investigated if the differences in carotenes accumulation induced by fruit bagging (Table 1) may be related to a differential transcriptional regulation of the MEP pathway. Analysis of transcript accumulation in the peel of NC and C fruits did not reveal substantial differences in expression of *DXS1*, *HDR* and *GGPPS* genes (Fig. 5). *DXS1* and *HDR* genes followed a similar expression pattern throughout ripening, with a transient induction in October and decreasing thereafter. The *GGPPS1* mRNA level increased progressively during ripening in both NC and C fruit, although in the last mRNAs accumulation was lower in November (Fig. 5). In addition to the genes described above, a second isoform of *DXS* and *HDR* as well as six others of *GGPPS* gene were analyzed. However, no differences were detected in the transcript levels between NC and C fruits (data not shown).

Effect of fruit bagging on transcript accumulation of genes involved in chromoplast differentiation in the peel of SR grapefruit

In order to elucidate if the accelerated rate of peel color change and the massive carotenoid accumulation that occurred in covered red grapefruit was associated with proteins involved in the differentiation of chromoplast and in carotenoid-sequestering structures, transcript levels of the genes fibrillin or plastid-associated proteins (*FIB* or *PAP*), orange (*Or*) and two sHSPs (*HSP20-4*, *HSP21*) were measured in the peel of NC and C fruits. A FIB (*FIB1-CitPAP*) has been previously described in citrus fruits (Moriguchi et al. 1998), and search on the *C. sinensis* genome database (www.phytozome.com) allowed the identification of a second putative FIB (FIB2, orange1.1g022196m.g), showing 31% of identity with FIB1 at amino acid level and 71% identity with

Table 1. Effect of fruit bagging on the composition of individual carotenoids (μg g^{-1} FW) in the peel of Star Ruby grapefruits. Immature green fruits were bagged in July, and non-covered (NC) and covered (C) fruits were harvested in October (69 days after bagging, dab), November (106 dab) or December (139 dab), corresponding to mature green, breaker and full color stages in NC fruit. The data are means of three determinations ± SE. [a]Mix of isomers; [b]Values lower than 0.05 μg g^{-1} FW.

	July	October		November		December	
Carotenoid	NC	NC	C	NC	C	NC	C
Phytoene	1.07 ± 0.18	6.98 ± 0.78	18.6 ± 1.69	11.0 ± 0.83	23.2 ± 0.51	12.8 ± 0.47	22.4 ± 0.06
Phytofluene	–	0.35 ± 0.01	4.03 ± 0.61	1.03 ± 0.14	6.13 ± 0.24	1.82 ± 0.12	6.20 ± 0.31
ζ-Carotene	–	–	–	–	0.22 ± 0.15	0.14 ± 0.03	0.14 ± 0.07
All-*E*-Lycopene	0.21 ± 0.04	1.27 ± 0.28	35.3 ± 2.0	1.37 ± 0.16	42.1 ± 0.83	0.92 ± 0.07	45.2 ± 0.24
β-Carotene	2.01 ± 0.03	2.00 ± 0.02	2.16 ± 0.05	2.03 ± 0.27	3.61 ± 0.01	0.69 ± 0.01	3.10 ± 0.21
α-Carotene	0.53 ± 0.05	0.95 ± 0.01	–	0.58 ± 0.09	–	–	–
Zeaxanthin	0.38 ± 0.06	0.46 ± 0.02	–	1.00 ± 0.18	–	0.27 ± 0.01	–
Violaxanthin[a]	5.76 ± 0.36	4.07 ± 0.24	0.99 ± 0.12	2.16 ± 0.38	0.84 ± 0.05	3.43 ± 0.12	0.86 ± 0.01
Neoxanthin[a]	1.39 ± 0.12	–	–	–	–	–	–
α-Cryptoxanthin	0.16 ± 0.01	–	–	[b]	–	[b]	[b]
Lutein	7.50 ± 0.12	5.62 ± 0.07	1.09 ± 0.01	3.07 ± 0.44	0.30 ± 0.06	0.44 ± 0.01	0.08 ± 0.01

Table 2. Effect of fruit bagging on ABA concentration (ng g^{-1} FW) in the peel of Star Ruby grapefruits. Immature green fruits were bagged in July, and non-covered (NC) and covered (C) fruits were harvested in October (69 days after bagging, dab), November (106 dab) or December (139 dab), corresponding to mature green, breaker and full color stages in NC fruit. The data are means of three independent replicates ± SE. [a]Significant differences between C and NC fruit at each harvest date ($P \leq 0.05$).

	ABA (ng g^{-1} FW)	
Date	NC	C
July	103 ± 12	–
October[a]	250 ± 14	83 ± 7
November[a]	742 ± 18	302 ± 25
December[a]	633 ± 17	187 ± 3

PAP-FIB of flax (*Linum usitatissimum;* Acc. JX_174449.1; Wang et al. 2012). *FIB1-CitPAP* transcripts abundance slightly increased, and *FIB2* remained constant with minor changes during ripening. However, in both genes, no differences in the expression level between C and NC fruit were observed (Fig. 6).

sHPSs are chaperones with functions related to membrane stabilization or prevention of protein aggregation (Al-Whaibi 2011). Some of these proteins accumulated during fruit ripening and have been associated with carotenoid accumulation (Neta-Sharir et al. 2005). In this work, two citrus members of sHSP potentially involved in chromoplast differentiation were identified: HSP20-4 and HSP21. Citrus *HSP20-4* presented an 83% identity with the orthologs from *Theobroma cacao* and 72% identity with *A. thaliana* class I *HSP* (AT1G07400; Theologis et al. 2000) and the *HSP21* of the flower *Cleome spinosa* (EU162609.1). *HSP21* is most likely the citrus orthologs of *HSP21* from tomato (38%), and the corresponding protein has been identified in the proteome of citrus fruit chromoplast (Zeng et al. 2011). Interestingly, both *HSP20-4* and *HPS21* genes showed a significant up-regulation (threefold and twofold, respectively) in C fruit from July to October. At that time, transcript levels of both *HSP* genes in the peel of NC fruits were almost similar to that of immature green fruits (July; Fig. 6). In light-exposed fruit, transcript levels of both genes increased in November and December, although with a different trend (Fig. 6).

The Or protein has been associated with differentiation of chromoplast from non-colored plastids in different plant tissues (Lu et al. 2006) and enhances accumulation of carotenoids (Li et al. 2001). An *Or* orthologous gene was identified in the sweet orange genome database (orange1.1g021509m), and its predicted protein presented a 76% identity with that of the *Or* gene from *Brassica oleracea* (ABH07405.1; Lu et al. 2006). Accumulation of *Or* transcripts was similar in the peel of NC and C grapefruit in October. The transcript level was progressively induced during ripening in NC fruits but remained stable in C grapefruits (Fig. 6).

Effect of fruit bagging on chloroplasts and chromoplast ultrastructure in the peel of SR grapefruit

The peel of SR fruit in July presented the characteristic chloroplasts of photosynthetic tissues, with stacked grana formed by well-organized thylakoids, as well as starch grains (Fig. 7A). However, in October, important differences in the ultrastructure of the chromoplast were detected between NC and C fruit. NC fruit plastids still maintained well-organized thylakoids stacked into grana and some plastoglobuli could be observed

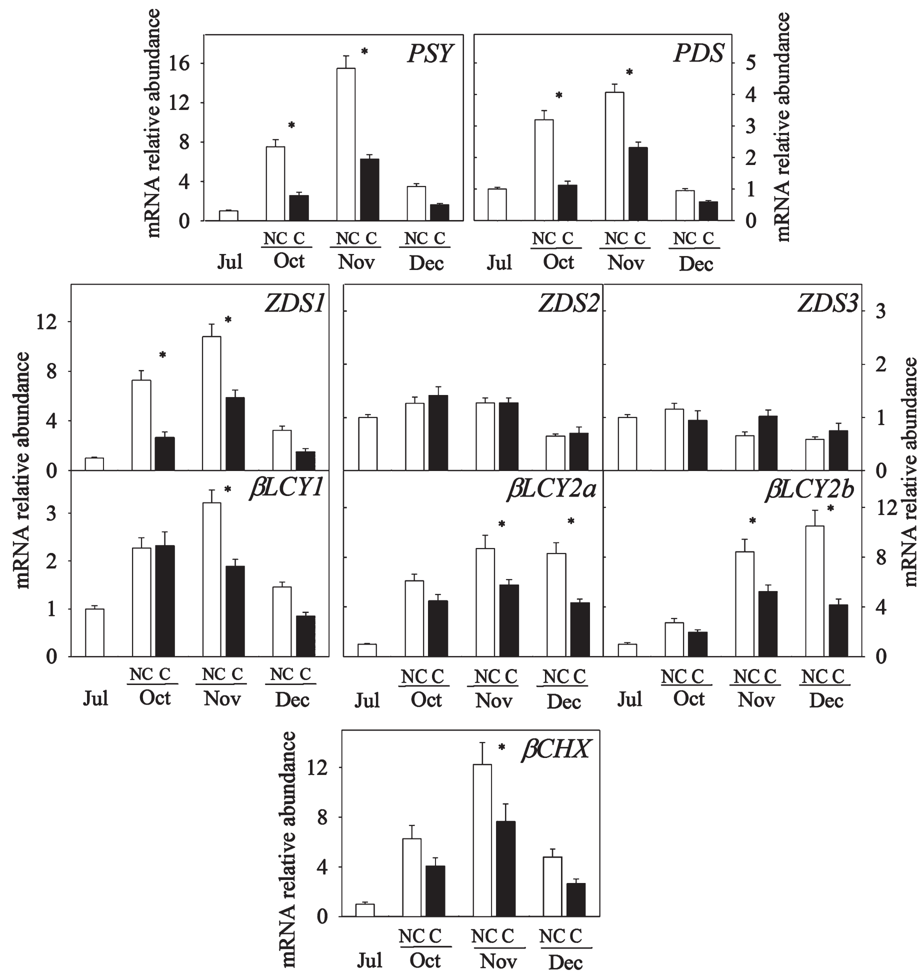

Fig. 4. Effect of fruit bagging on the expression of carotenoid biosynthetic genes in the peel of Star Ruby grapefruits. Immature green fruits were bagged in July, and non-covered (NC) and covered (C) fruit were harvested in October (69 days after bagging, dab), November (106 dab) or December (139 dab), corresponding to mature green, breaker and full color stages in NC fruit. The genes analyzed were phytoene synthase (*PSY*), phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS1*, *ZDS2* and *ZDS3*), lycopene β-cyclase 1 (*βLCY1*), lycopene β-cyclase 2 (*βLCY2a* and *βLCY2b*) and β-carotene hydroxylase (*βCHX*). The data are means ± SE of four replicates. Asterisks indicate significant differences between C and NC fruit at each harvest date ($P \leq 0.05$).

(Fig. 7B), whereas the photosynthetic membranes of C fruit appeared to be disassembled with only a few grana and thylakoids visible (Fig. 7C). Moreover, carotenoid storage structures, such as plastoglobuli and crystals, were observed after October inside the chromoplast of C fruit, whereas these structures were almost absent in NC fruit (Fig. 7B, C). In December, chromoplast of NC and C fruits contained round and even highly osmiophilic plastoglobuli, whereas the lycopene crystals and electron dense uneven structures were only observed in C fruit (Fig. 7D, E). These electron dense osmiophilic structures were highly variable in size (ranging from 80 to 500 nm wide and from 80 to 800 nm long). These structures were not found co-existing in the same plastid with typical plastoglobuli. The predominant structures in NC fruit were small, electron dense, spherical and even plastoglobuli together with the presence of non-photosynthetic membranes (Fig. 7D). It is important to note that no significant differences in chromoplast number or size were detected between NC and C fruit during ripening (data not shown).

Discussion

Coloration of citrus fruit is an important quality parameter directly affecting consumer preference and is strongly influenced by environmental and endogenous factors (reviewed by Kato 2012, Rodrigo et al. 2013). Light is recognized as one of the most important environmental

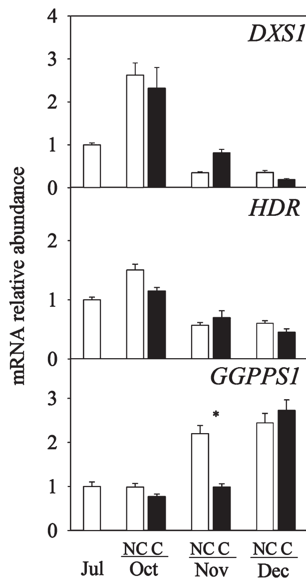

Fig. 5. Effect of fruit bagging on the expression of the principal genes of the MEP pathway in the peel of Star Ruby grapefruits. Immature green fruits were bagged in July, and non-covered (NC) and covered (C) fruits were harvested in October (69 days after bagging, dab), November (106 dab) or December (139 dab), corresponding to mature green, breaker and full color stages in NC fruit. The genes analyzed were 1-deoxy-D-xylulose-5-phosphate synthase (*DXS1*), hydroxymethylbutenyl diphosphate reductase (*HDR*) and geranyl geranyl pyrophosphate synthase (*GGPPS1*). No significant differences were detected between C and NC fruit at each harvest date ($P \leq 0.05$).

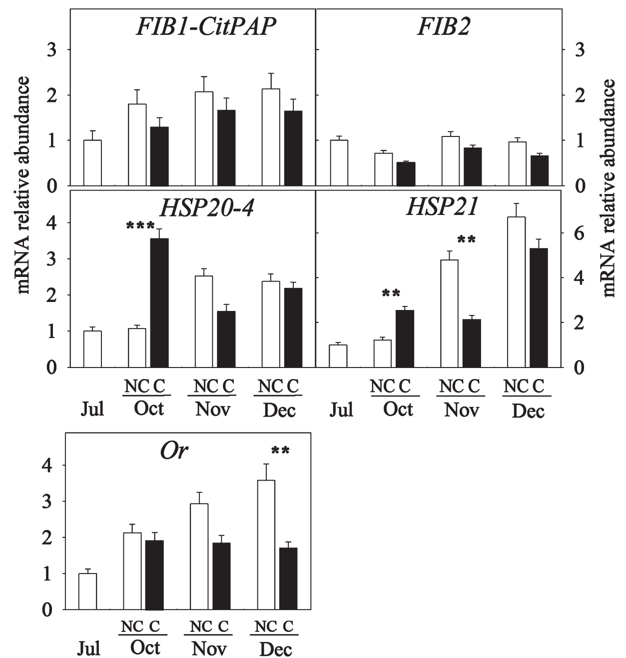

Fig. 6. Effect of fruit bagging on the expression of fibrillins (*FIB1-CitPAP* and *FIB2*), orange (*Or*) and small heat shock proteins (*HSP20-4* and *HSP21*) genes in the peel of Star Ruby grapefruits. Immature green fruits were bagged in July, and non-covered (NC) and covered (C) fruits were harvested in October (69 days after bagging, dab), November (106 dab) or December (139 dab), corresponding to mature green, breaker and full color stages in NC fruit. Asterisks indicate significant differences between C and NC fruit at each harvest date (**$P \leq 0.01$, ***$P \leq 0.001$).

factors stimulating citrus peel coloration during natural ripening. In oranges and mandarins, it is frequently observed that fruits located inside the tree canopy or the internal side of fruits not exposed to light display lighter coloration than those directly exposed to light (Cronje et al. 2011, 2013). A similar stimulatory effect of light on carotenoid accumulation has been also described in tomato fruit (Azari et al. 2010). It is, therefore, paradoxical that the peel of SR grapefruit grown under natural shading, i.e. covered by leaves or inside the tree canopy, develops a more intense red coloration (Fig. 2, Fig. S1). Moreover, this observation in red grapefruit seems unrelated to differences in agronomical practices as it occurred similar in Northern and Southern hemisphere orchards, with profoundly different environmental conditions. It is interesting that exceptions to this light-inductive effect on coloration have also been described in specific storage plant organs that usually grow under darkness, such as carrot roots (Fuentes et al. 2012, Rodriguez-Concepcion and Stange 2013).

Light avoidance of SR grapefruit during ripening (by covering fruit with plastic bags at immature green stage) accelerated peel color development compared to light-exposed fruit (Fig. 3). This effect was accompanied by a higher rate of Chl degradation, massive accumulation of carotenes and a reduction of xanthophylls as well as downstream products such as ABA (Fig. 3, Tables 1 and 2). Moreover, total carotenoid content remained almost constant in the peel of fruit exposed to normal photoperiodic conditions, whereas fruit grown under darkness registered a fourfold increment with the most noticeable change occurring at an early stage of fruit ripening, from July to October (Fig. 3, Table 1). Despite the absence of changes in total carotenoids in light-grown fruits, the carotenoid profile was profoundly affected, showing a significant increase in the linear carotenes phytoene and phytofluene and, to a lesser extent, in lycopene (Table 1). Absence of light promoted an increased accumulation of lycopene which was also accompanied by accumulation of phytoene and phytofluene (Table 1). Therefore, in October, linear carotenes represented more than 90% of total carotenoids in C fruit, compared to only 37% in NC (Table 1). This dark-induced alteration in carotenoid profile suggests the accentuation of the bottleneck at lycopene cyclization level in the carotenoid pathway that was described to exist in the red grapefruit compared

to oranges or white grapefruits (Alquézar et al. 2009, 2013). In the present study, the absence of light seems to potentiate this blockage as suggested by the higher concentration of lycopene and upstream carotenes and the lower levels of downstream metabolites (xanthophylls and ABA) in C fruit compared to NC fruit (Tables 1 and 2).

This increase in linear carotenes in the peel of C SR fruits was only partially mirrored by changes in the transcript accumulation of carotenoid biosynthetic genes. The most important changes in carotenoid composition occurred between July and October, when *PSY*, *PDS* and *ZDS* transcripts levels were higher in NC fruit than in C fruit (Fig. 4). In light-growing conditions, the expression of the carotenoid biosynthetic genes, mainly *PSY*, *PDS*, *ZDS1* and *βLCY2*, was markedly up-regulated in the peel of SR fruit, which is similar to that reported in other fruits like tomato (Gupta et al. 2014) and vegetative tissue of *A. thaliana* (Toledo-Ortiz et al. 2010). Despite this fact, it is worth noting that the relative low transcripts levels of key carotenoid biosynthetic genes under darkness may be enough to allow a major lycopene accumulation in the peel of C fruit (Table 1). These results indicate that the activity of upstream steps in C fruit might be sufficient to provide an important flux of intermediates into the pathway and therefore, an elevated lycopene production. However, the lycopene *β*-cyclase activity should not be enough to cyclize this carotene effectively, which is supported by the lower downstream products (xanthophylls and ABA) found in C fruit (Tables 1 and 2). The *β*LCY2 enzyme has been described as a limiting step in the carotenoid biosynthetic pathway in SR grapefruit due to reduced gene expression that contributes to lycopene accumulation in the pulp compared to other *Citrus* species or grapefruit varieties (Alquézar et al. 2009, 2013, Mendes et al. 2011, Costa et al. 2012). It may, therefore, be suggested that the reduced increase in the transcript accumulation of *βLCY2* in dark-grown fruits may contribute to enhance lycopene content in this tissue.

Light did not affect mRNA levels of the key genes from the MEP pathway in SR grapefruit (Fig. 5), the main source of precursors for carotenoid biosynthetic pathway. It has been reported that light has a positive effect on the MEP pathway (Botella-Pavía et al. 2004); however, in SR grapefruit, light does not seem to play an important role in the transcriptional regulation of these genes (Fig. 5). Despite the absence of differences in transcript levels of genes from the MEP pathway, it cannot be ruled out the possible existence of greater substrate availability under dark conditions due to reduced competition with other biosynthetic pathways. For example, it is well established that under dark conditions, Chl degradation is accelerated but Chl biosynthesis is also impaired; carotenoids and Chl biosynthetic pathways share common precursors (Hemmerlin et al. 2012), thus the reduced demand of substrates for Chl biosynthesis could result in greater availability for carotenoid biosynthesis when light levels are reduced.

The peel of shaded fruits showed an accelerated conversion of chloroplasts into chromoplast compared to those growing under light regime. This situation imposed remarkable changes in plastids ultrastructure and favored the early appearance of structures to accumulate carotenoids (Fig. 7). Hence, the increase in the capacity to sequester newly synthesized carotenoids concomitant with the development of new biosynthetic sites in chromoplast, the plastoglobuli (Ytterberg et al. 2006), could be explaining the massive carotenoid accumulation in covered SR fruit. Plastoglobuli have been suggested to be involved in chromoplast biogenesis being also an active site for carotenoid biosynthesis (Ytterberg et al. 2006) and accumulation (Nogueira et al. 2013). Thus, the earlier presence of plastoglobuli in C fruit compared to NC could be contributing to the higher carotenoid content in C fruit (Fig. 7). The importance of adequate structures for carotenoid biosynthesis and accumulation has been demonstrated since defects in plastid development results in impaired accumulation of carotenoids. Thus, a notable increase in storage capacity may explain the burst in the accumulation of carotenoids by enhancing sink strength in different organs (Li et al. 2001, Li and Van Eck 2007, Kim et al. 2013). An illustrative example of how light can directly affect carotenoid accumulation through modification of plastids ultrastructure has been described in carrot roots developed under light. In this tissue, differentiation of leucoplasts into chloroplasts instead of chromoplast prevented the typical burst in carotenoid accumulation that occurs under normal conditions (darkness) (Rodriguez-Concepcion and Stange 2013). These results agree with the idea that the formation of chromoplast is an important 'driving force' regulating carotenoids accumulation in plants. It has also been shown that the heterologous overexpression of the *Or* gene, which is related to chromoplast differentiation in several plant species, leads to massive accumulation of carotenoids without affecting the transcript levels of main biosynthetic genes (Li et al. 2001, Kim et al. 2013). Similar results were obtained in rice callus, where chromoplast differentiation is triggered either by a direct expression of *Or* gene or by increasing the flux through the carotenoid pathway (Bai et al. 2014). Thus, organelle biogenesis is a determinant of the storage capacity of the plastids and can directly affect carotenoid accumulation by providing a larger sink (Cazzonelli and Pogson 2010). From our results, it is difficult to establish whether abnormal carotenoid and

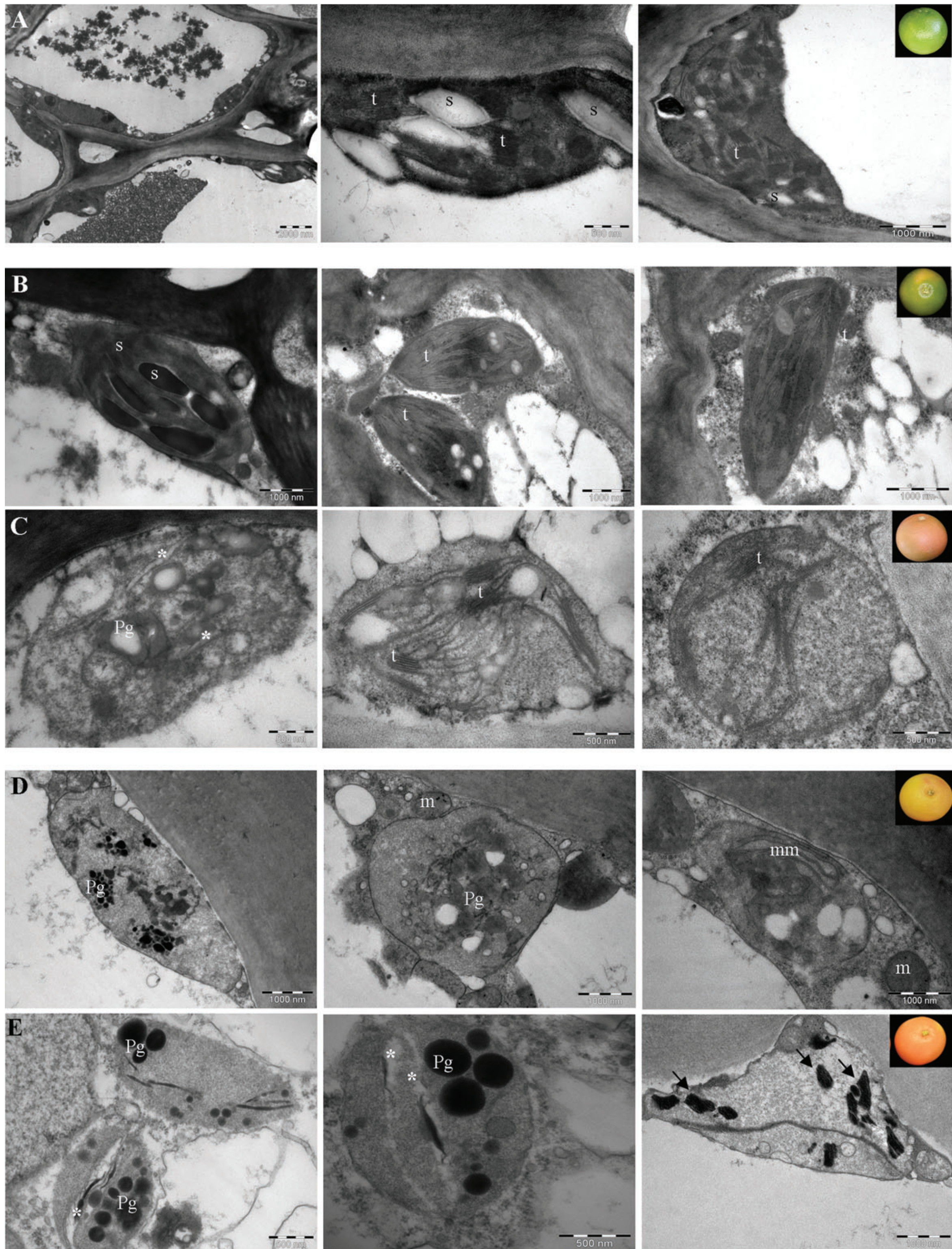

Fig. 7. Transmission electron microscopy images of chloroplasts and choromoplast in the peel of Star Ruby grapefruit in July (A) and in the peel of non-covered (NC) or covered (C) fruit harvested in October (69 days after bagging, dab) (B and C, respectively), and December (139 days after bagging) (D and E, respectively). mm, membranes; m, mitochondria; Pg, plastoglobulus; s, starch granules; t, thylakoids. *Lycopene crystals. Black arrows point to uneven electron dense structures detected only in covered fruit.

lycopene accumulation in covered SR fruits is linked to altered chromoplast differentiation as a result of reduced light or alternatively, may be a consequence of a modification in the balance of carotenoid metabolism.

The transcript accumulation of genes coding for proteins that are induced during differentiation of chloroplasts in to chromoplast, such as of *FIB*s, *Or* and small *HSP*s genes (Neta-Sharir et al. 2005, Lu et al. 2006), has also revealed differences between NC and C fruits. Among the genes investigated, only *HSP20-4* and *HSP21* were specifically stimulated in dark-grown fruits (Fig. 6) and, interestingly, this was coincident with advanced chromoplast differentiation of those fruit in October (Fig. 7C). Nevertheless, the function of these HSPs in SR peel appears to be related to chloroplasts to chromoplast conversion and not to the massive lycopene accumulation in these organelles, because similar transcript levels were detected in both fruits in November. This coincides with the onset of chromoplast differentiation in light-grown fruits (Fig. 7), which presented a relative low content of lycopene (Table 1). Recent work described the relevance of these proteins as cofactors in the targeting of proteins to the plastid outer envelope to favor plastid differentiation and development (Kim et al. 2011). Similarly, *HSP21* was also described to be induced in tomato fruit during ripening (Neta-Sharir et al. 2005), and its transcript abundance was higher in cassava genotypes that accumulated high amounts of β-carotene compared to the white varieties, suggesting a specific role of this protein on carotenoid sequestration (Carvalho et al. 2012). Regarding citrus fruit, these sHSPs have also been reported to be induced in lycopene-accumulating citrus mutants like ‘Cara Cara’ sweet orange (Pan et al. 2012) and were suggested to be relevant to the ripening process, as it is of greater abundance in the plastid proteome of sweet orange (Zeng et al. 2011). FIBs proteins were also proposed to play a role in maintaining structural integrity of the plastoglobuli and their overexpression in tomato fruit, leading to an increase in carotenoid accumulation and to a new cluster arrangement of the plastoglobuli (Simkin et al. 2007). However, chloroplasts to chromoplast conversion in SR grapefruit does not seem to be related to FIB mRNA levels (*FIB1-CitPAP* and *FIB2*) (Fig. 6), thereby opposing what has been described in tomato and pepper fruit (Simkin et al. 2007). In contrast, in the lycopene-accumulating orange mutant ‘Hong Anliu’, a higher expression of *CitPAP* was described compared to the parental variety (Pan et al. 2009), which was not observed in SR grapefruit (Fig. 6), and thus suggesting the existence of different mechanisms between oranges and grapefruits in the metabolic process leading to lycopene accumulation. Similarly, the transcript level of *Or* gene showed no relevant changes during ripening in the absence of light, although in fruits grown in light conditions, its expression showed an increment during ripening (Fig. 6). Therefore, its function may be related to chromoplast division and mediation in proteins interactions (Li et al. 2001, Lu et al. 2006) that may be specially required under light conditions.

The experimental system used in this work revealed that high lycopene accumulation in SR fruit grown under darkness is tightly associated with changes involving the acceleration of chromoplast ultrastructural differentiation, resembling the process of green tissue senescence. In this sense, a premature chromoplast differentiation may provide a higher biosynthetic capacity and sink for carotenoid accumulation without necessarily involving major transcriptional changes in the carotenogenic genes. The transcripts accumulation induction of most upstream carotenoid biosynthetic genes that normally occur in citrus fruit during maturation (Rodrigo et al. 2004) was also observed under dark conditions with maximal expression in November, which may sustain carotenoids accumulation in a global scenario of high substrate availability and an increased storage capacity in C fruits. A similar situation has been described in the regulation of carotenogenesis in carrot roots, where the massive accumulation of carotenoids in dark-grown roots was only partially correlated with the expression of carotenogenic genes, suggesting that additional mechanisms are also crucial in determining carotenoid content and composition in this organ (Rodriguez-Concepcion and Stange 2013). It is important to emphasize the notion that in SR grapefruit lycopene cyclization is highly reduced due to a lower expression and less active *βLCY2* enzyme in red grapefruit compared to other citrus cultivars (Alquézar et al. 2009, Alquézar et al. 2013). Hence, in this scenario, reduced expression of all *βLCYs* in the peel of dark-grown fruits (Fig. 4) may potentiate the bottleneck at the lycopene cyclization step in the pathway promoting massive accumulation of lycopene and upstream carotenes. This blockage of lycopene cyclization in fruit developing in the absence of light is further supported by the reduced amounts of xanthophylls and, more specifically, of the downstream phytohormone ABA. Thus, in SR fruit, light signals seem to regulate carotenoid accumulation at the molecular and structural level by influencing both biosynthetic capacity and sink strength. Furthermore, this work supports with the hypothesis that carotenoid accumulation can be boosted by triggering the synthesis of a plastid deposition sink to allow their enhanced biosynthesis and storage.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Sequences of the primers used for real-time qPCR.

Fig. S1. Effect of natural shading on SR fruit peel coloration.

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